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AN AUTONOMIC NERVE PLEXUS CONTROL OF THE GALL BLADDER IN MYXINE*

BY

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1. INTRODUCTION

Experiments with electrical stimulation of the vagus nerve have clearly demonstrated that the vagus nerve in *Myxine* is motor to the branchial sacs and the striated muscles of the constrictor cardiae (see NICOL 1952). As regards the intestine the view has been expressed that also in this case the effect is motor although experimental evidence in favour of this opinion is very tenuous (PATTERSON & FAIR 1933, FÄNGE 1948). From a morphological point of view there is a curious disproportion in the size of the extremely thin smooth musculature layer of the intestine on the one hand and that of the intestinal ramus, composed of a large number of very thin fibres and very numerous ganglion cells, on the other hand.

The gall bladder in *Myxine* is a comparatively large organ in conformity with the large size of the bilobated liver. The musculature of the gall bladder is stronger than that of the intestine, which ought to give an opportunity for good observations in experiments performed in order to elucidate the function of the vagus intestinalis. However, the innervational system of the gall bladder was not known previously and a study of this structure was, thus, a prerequisite for further experiments.

* Aided by grants from the Swedish Natural Science Research Council.

2. METHODS

For anatomical and histological purposes a number of 10—15 cm long specimens of Myxine glutinosa were collected at Kristineberg, on the west coast of Sweden. The fixatives were Bouin's fluid and Bodian's fixative No. 2 (Romeis 1948). Portions of the body comprising the caudal part of the branchial region and the anterior part of the trunk region were sectioned transversally and sagittally. The thickness of the sections was 10—15 microns. The staining was performed according to Bodian (1936, see also Romeis 1948) or Palmgren (to be published in this Journal).

In the experiments large specimens were used. The animals were decapitated or anaesthetized with urethane and nailed to a dissection board. The liver region with the gall bladder and the branchial region were exposed. Stimulation was given by means of a couple of silver electrodes from a thyratron stimulator (frequency: 20 impulses per second; length of impulses 10 milliseconds; 5 volts). In the stimulation experiments the vagus nerve of each side was cut off at the central end in order to avoid the effect produced by stimulation of afferent vagal fibres.

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3. THE GALL BLADDER AND ITS INNERVATIONAL SYSTEM

The wall of the gall bladder consists of an epithelium, a connective tissue layer, a layer of smooth musculature, a connective tissue layer and a peritoneal covering (see Maas 1896, Cole 1913). There are conflicting opinions on the structure of the muscular coat of the gall bladder in the literature. This may, at least in part, be explained by the fact that the musculature varies in thickness in different parts of the bladder and may be altogether deficient in some places. In general, however, the smooth muscular layer is strong. In some places there seem to be three layers of smooth muscle cells with an alternating orientation of the long axies of the cells between the layers. Mostly the musculature is composed of only one (Fig. 1) or two layers. Generally, the middle portion of the bladder is dominated by circular muscle cell elements arranged transversally to the long axis and the anterior and posterior portions have muscular fibres which are mostly more or less longitudinal in arrangement.

There seems to be a slight thickening in the musculature around the mouths of the anterior and posterior bile ducts. This is more pronounced around the entrance into the ductus choledochus. The existence of some mechanism which is able to keep the bile in the bladder seems to us to be indicated by the observation made by Cole (1913, p. 326) that no bile is lost if the ducts are cut off and the bladder is extracted from its place during preparative work.

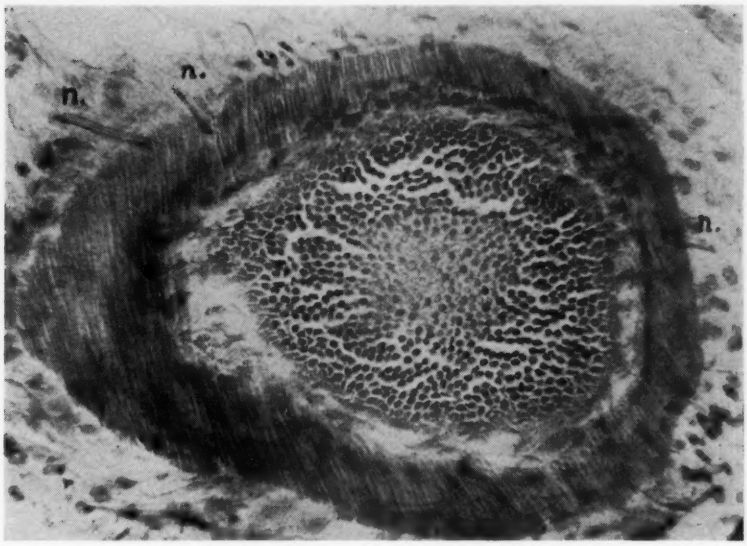

Fig. 1. Tangential section through the anterior end of the gall bladder of Myxine. In the centre of the picture the epithelium of the bladder wall. Between this and the smooth musculature a space filled with loose connective tissue. Note small nerves (n.) situated just outside the layer of muscular cells. Microphotograph, 200 ×.

We have been able to confirm this in our own observations. The ductus choledochus connects with the bladder at a very acute angle and in this way a sort of valve is formed by the thin lip forming the vertex of the acute angle. The same is the case with the posterior and anterior bile ducts. The latter forms, when in an undisturbed position, a very characteristic U-shaped loop which is open anteriorly and connects with the gall bladder wall obliquely from behind (Fig. 2). In preparations this loop disappears due to stretchings caused by dislocations (see e.g. MARINELLI & STRENGER 1956, Fig. 107). The part of ductus choledochus which is located within the tissue of the intestinal wall is surrounded by a number of nodules of cells which by MAAS (1896) and COLE (1913) have been denoted as pancreatic tissue.

The wall of the gall bladder, the ductus hepaticus anterior and posterior, and the ductus choledochus are strongly innervated. In the place where the two intestinal branches of the vagus nerve, one on each side, reach the wall of the oesophagus and penetrate through the coat of striated muscles of the constrictor cardiae, the hepatic nerves branch from the main intestinal nerves which here form a plexus in the striated musculature. In a caudal and slightly ventral direction the hepatic nerves, one or two on each side of the oesophagus, run to a place on the ventral surface of the intestine immediately in front of the ductus choledochus (Fig. 2). The left hepatic nerve or nerves closely

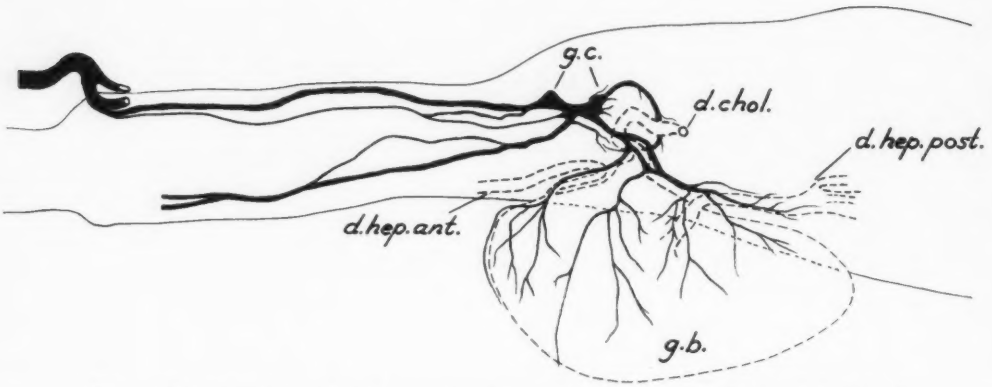

Fig. 2. Reconstruction of the innervational system of the gall bladder and hepatic ducts of Myxine. Ventral view. The entrance of the left vagus intestinalis shown in the figure. The lumina of the gall bladder and hepatic ducts shown by broken lines. The outline of the intestinal canal is indicated. d.chol. — ductus choledochus. d.hep.ant. — ductus hepaticus anterior. d.hep.post. — ductus hepaticus posterior. g.b. — gall bladder. g.c. — large accumulations of ganglion cells. 20 ×.

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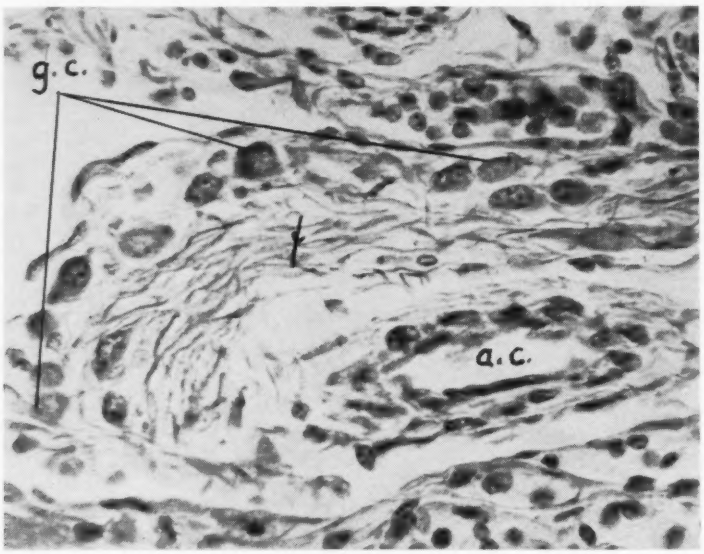

Fig. 3. Section through one of the ganglion cell accumulations close to the ductus choledochus of Myxine. The ganglion cells (g.c.) seen in the section situated to the left of and above the bundle of fibres (f) of the hepatic plexus. a.c. — arteria coeliaca. Microphotograph, 440 ×.

follow the course of the arteria coeliaca (see e.g. MARINELLI & STRENGER 1956). In the vicinity of the ductus choledochus the nerves from the two sides unite for a short distance and then split up repeatedly again. In this place one or more aggregations of ganglion cells occur (Fig. 3). In the vicinity of the

ductus choledochus and around the covering of pancreatic tissue the nerve branches contain other aggregations of ganglion cells. These ganglion cell aggregations form a structure which may be termed the plexus hepaticus. In this region a number of about 400 ganglion cells have been counted in a 140 mm long specimen. Ganglion cells seem to be completely absent in the hepatic nerves in front of this place.

The ganglion cells and nerve fibres of the plexus are to a large extent located close to the pancreatic tissue covering of the ductus choledochus. There are, however, no structures which give further information as to whether this close relationship between the nervous elements and the pancreatic tissue is more than incidental. The vast majority of the nerves which run peripherally from the hepatic plexus are in any case directed towards the wall of the gall bladder. In this the nerves spread over the exterior surface of the muscular wall (Fig. 1). A few small branches follow along the ductus hepaticus anterior and posterior. Ganglion cells are absent or very rare in these branches situated peripherally to the hepatic plexus.

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Previous investigations have indicated that there is no autonomic innervation of the intestine in *Myxine* besides the vagus (MÜLLER 1838, BRANDT 1922). The present investigation of the hepatic innervation is in conformity with this opinion.

4. ELECTRICAL STIMULATION OF THE NERVES TO THE GALL BLADDER

A strong contraction of the gall bladder was obtained when the left or right ramus intestinalis of the vagus nerve was stimulated in the gill region. The same response was obtained when the electrodes were placed close to the ductus choledochus or at the wall of the gall bladder near the ductus choledochus. A small piece of a filter paper moistened with 0.5 % or 1.0 % nicotine solution was placed in contact with the ductus choledochus and the plexus hepaticus. A few minutes later stimulation of the intestinal branches of the vagus nerve still resulted in powerful contractions of the gill sacs but the gall bladder no longer reacted.

During the vagal stimulations the intestine was carefully observed. Different arrangements were made in order to detect small movements. However, no movements of the intestine could be noticed. This is in accordance with FÄNGE (1948).

5. THE EFFECTS FROM ACETYLCHOLINE AND ADRENALINE

Attempts were made to record on a smoked drum movements of isolated strips of the gall bladder (sea water diluted with ¼ distilled water was used

as suspension fluid) and extremely small movements were observed by addition of acetylcholine. Due to the small size of the responses this recording method was abandoned. Instead in situ experiments and observations with the naked eye were made. Application of small drops of acetylcholine solutions on the surface of the gall bladder (conc. 10^{-4} g/ml) produced as strong contractions of the gall bladder as electrical stimulation of the vagus nerve. Adrenaline (conc. 10^{-4} g/ml) seemed to have a relaxing effect. No other drugs were tried.

6. DISCUSSION

The simultaneous existence of a well-defined smooth muscle layer in the wall of the gall bladder and an innervational system formed exclusively from the vagus nerve strongly indicates that the nerve is motor to the bladder musculature. This is clearly demonstrated to be the case by the experiments with electrical stimulation of the vagus nerves. The blocking effect produced by application of a nicotine solution to the hepatic plexus during these experiments may indicate that the motor pathways to the gall bladder form synapses in the plexus. Probably the vagal fibres to the gall bladder are cholinergic because acetylcholine has a contracting effect on the smooth muscles of the gall bladder.

Thus the reactions of the musculature of the gall bladder to acetylcholine and adrenaline are the same as those of the intestinal muscularis (FÄNGE 1948, EULER & ÖSTLUND 1956). However, electrical stimulation of the vagus nerve does not cause visible movements of the intestinal musculature (FÄNGE 1948). During the stimulation experiments when strong contractions of the gall bladder were obtained the behaviour of the intestine was also carefully observed with a negative result. According to PATTERSON & FAIR (1933) electrical stimulation in *Polistotrema* caused relaxation of the intestine but this reaction was very indistinct. It is possible that the negative results of electrical stimulation in our experiments were caused by a detrimental effect of a too high temperature. The experiments were performed at room temperature (18°C). In other experiments with intestinal and striated muscles of Myxine we have observed that the preparations are more reactive at 10°C than at 18—20°C.

Very likely the vagus nerve plays a motor role in an emptying reflex mechanism of the gall bladder. In lower vertebrates the information regarding such a mechanism is deficient (see NICOL 1952). In higher vertebrates there is a dual innervation of the gall bladder comprising sympathetic as well as vagal (parasympathetic) nerves (see e.g. KUNTZ 1947) which controls the cooperation of the sphincter musculature of the ductus choledochus and the musculature of the wall of the gall bladder. Although this complex mechanism is absent in *Myxine* there are indications that the accumulation and emptying

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of the bile of the gall bladder is affected not only by the contractions of the wall. It seems that the valves of the ducts serve to keep the bile in the bladder. This may be a passive effect when the musculature of the wall is relaxed. When the musculature contracts the valves may be automatically opened.

7. SUMMARY

1. The wall of the gall bladder of Myxine has a comparatively strong muscular layer. The ductus hepaticus anterior and posterior as well as the ductus choledochus connect with the wall of the bladder at a very oblique angle which forms a kind of valve at the mouths of these ducts.

2. The gall bladder and the bile ducts of Myxine are innervated exclusively by hepatic branches from the intestinal rami of the vagus nerves.

3. In the vicinity of the ductus choledochus and the pancreatic tissue surrounding parts of this duct there are aggregations of ganglion cells which form a hepatic plexus. Ganglion cells are absent in the hepatic nerve branches anterior to this plexus and rare in the nerve branches which innervate the gall bladder peripherally to this hepatic plexus.

4. Electrical stimulation of the vagus nerve has demonstrated that this innervational system is motor to the gall bladder musculature. Experiments with nicotine during electrical stimulation indicate the presence of synapses in the hepatic plexus.

5. The musculature of the gall bladder reacts to acetylcholine and adrenaline in the same way as the intestinal musculature. Acetylcholine produces contraction and adrenaline relaxation.

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ANATOMICAL AND PHYSIOLOGICAL STUDIES ON THE ENTERON OF LAMPETRA FLUVIATILIS (L.)*

BY

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1. INTRODUCTION

Recently it has been demonstrated that in *Lampetra planeri* the alimentary canal is innervated exclusively from the vagus nerve apart from a portion close to the cloaca. In this posterior part the intestinal wall receives an additional innervation from spinal fibres entering from the posterior end of the alimentary canal (JOHNELS 1956). This difference in innervation may imply dissimilar physiological properties of the anterior and posterior portions of the intestine in lampreys which have not previously been studied in this respect.

2. ANATOMICAL RESULTS

It is generally considered that the rectal portion of the alimentary canal in *Lampetra* is only a very short transitional tube conducting from the intestine to the cloaca. This portion is situated within the covering of the striated muscles of the nodus muscularis cloacalis (see MARINELLI and STRENGER 1954). KRAUSE (1923) described the structure of the wall of this rectal portion which is different from that of the intestine in the absence of a spiral valve, in a gradual reduction of the size and number of the folds of the mucosa, and in the increase of the thickness of the smooth musculature (see also JACOBSHAGEN 1931). MARINELLI and STRENGER (1954) state that different parts of the alimentary canal in *Lampetra fluviatilis* cannot be anatomically distinguished.

* Aided by grants from the Swedish Natural Science Research Council.

However, in specimens of *Lampetra fluviatilis* caught in the autumn in fresh water a portion with a length of 1.5—2 cm in front of the rectal part which is covered by the nodus muscularis cloacalis is distinctly thicker than the part of the intestine situated in front of this portion. This caudal part has a stronger muscular layer in the wall than the rest of the intestine.

The muscular layer of the main part of the intestine is weakly developed. Nevertheless it forms an almost complete layer of mostly circular elements close to the peritoneal lining and covers the vascularized and haemopoietic tissue which forms the main part of the wall as well as of the folds and of the spiral valve. However, a large number of the muscle fibres are oriented more or less obliquely in relation to the circular fibres and there are, also, several bundles of longitudinal muscles.

The thickness of the muscular layer is very markedly increased in the caudal part of the alimentary canal (Fig. 1). In this part the lymphatic tissue is very sparse; the fact that this part in spite of this is thicker than the intestine in front of it is due to the relatively very strong musculature. This smooth musculature is predominantly circular. However, also more or less longitudinal muscle bundles are present, and particularly in the part situated within the striated muscular cap there are a number of quite thick longitudinal muscle bundles (see KRAUSE 1923). In the wall of this caudal portion there is, further, an increased number of small arteries as compared to the anterior portion of the intestine. These arteries arrive at the enteron through small remnants of a dorsal mesenterium as demonstrated by JACOBSHAGEN (1931, Figs. 9—11). Of the venae of this portion a number are widened in a sinusoid manner.

In *Lampetra* the main part of the intestine lacks a mesenterium. The part situated within the nodus muscularis cloacalis—the rectum—has a complete dorsal mesenterium. In front of this portion there are—as mentioned—small remnants of a dorsal mesenterium. Within this portion in front of the rectum which is marked by the presence of these mesenterial remnants the spiral valve gradually disappears in a caudal direction, the folds of the mucosa grow smaller towards the rectum, the haemopoietic tissue characteristic of the intestine is absent, and the muscular layer is obviously thicker than in the intestine proper. This portion—a coprodaeum (see JACOBSHAGEN 1937)—which is about 1.5—2 cm long in adult specimens of *Lampetra fluviatilis* is, thus, a transitional portion between the intestine and the rectum. The latter, situated within the nodus muscularis cloacalis, is only about 0.5 cm long.

In the experiments in the present work this transitional portion has been tested together with the rectum. Both portions are characterized by the thick layer of smooth musculature and the additional spinal innervation and are together subsequently termed the rectal piece or portion.

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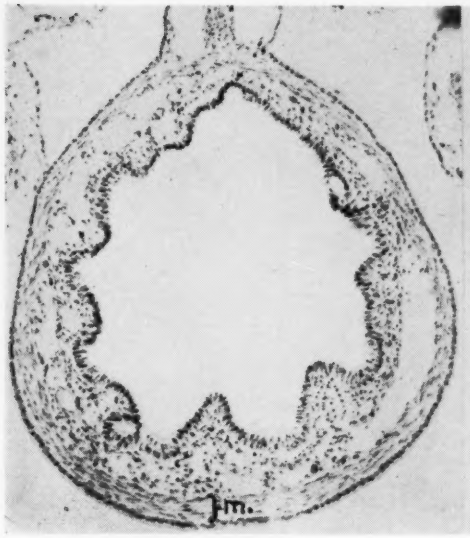
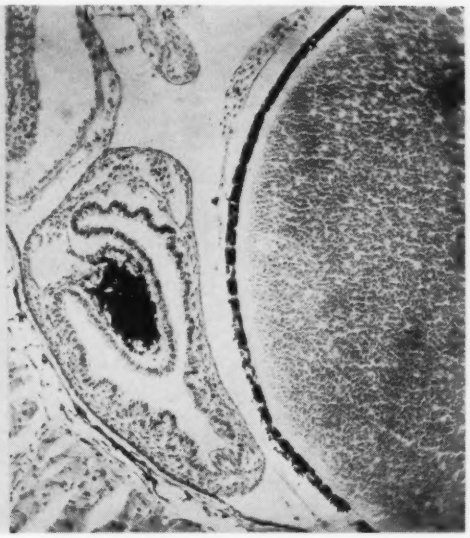
A.   B.

Fig. 1. Photomicrographs of transverse sections in adult female of *Lampetra planeri*. 80 ×. A. Rectal portion. Below the peritoneal covering, seen as a row of nuclei, the circular muscle layer (m.). Dorsally the mesenterium. B. The alimentary canal 6 mm in front of A. The layer of smooth musculature is very thin. The centre of the spiral valve is largely occupied by pigment. To the right an almost ripe egg.

3. EXPERIMENTAL RESULTS

The experimental material consisted of pieces of the alimentary canal of *Lampetra fluviatilis*. Because of the very gracile features of the intestinal tract preliminary tests were performed on pieces placed in a bath of Ringer solution in a Petri jar. The test substances were added to the bath in the Petri jar. After each experiment the bath solution was replaced by fresh Ringer solution and the preparation was ready for a new experiment after a rest of ten minutes. Pieces of a length of 1.5 cm of the intestine and rectal pieces of the same length were also placed in a bath of Ringer solution, one end being fixed and the other end attached to a very light frontal-drawing lever which recorded the contractions on a slowly moving smoked drum. In this case the bath was continuously oxygenated.

From the experiments it was evident that the intestine apart from the rectal piece showed reactions which were generally extremely weak. The middle part of the intestine showed no visible effect when treated with 5-Hydroxytryptamine (5-HT), Histamine and Acetylcholine (Ach) in concentration 1×10^{-6} —1×10^{-4}. After pretreatment with physostigmine 1×10^{-6} for ten minutes a slight effect was recorded with Ach (1×10^{-4}).

In contrast to this the rectal piece showed very distinct reactions from treat-

ment with 5-HT ($\frac{1}{2} \times 10^{-5}$). The reaction was strong peristaltic contractions of the prevailing circular musculature (see above). Ach (1×10^{-4}) also showed a similar but much weaker effect than 5-HT. After pretreatment with physostigmine this effect of Ach was augmented; the effect of 5-HT was not influenced. Histamine, adrenaline and noradrenaline had no visible effect.

Because of the very slight contractions longitudinally of the intestinal and rectal parts a special technique was applied in the case of the rectal piece. It was cut transversally into ten small parts. These circular structures were tied together forming a chain. In this way the very obvious contractions of the mainly circular muscle elements were successfully recorded on the smoked drum.

4. CONCLUSIONS

The physiological reactions of the various parts of the intestine are in agreement with the fact that the rectal piece has a much stronger, mainly circular, musculature than the other parts of the alimentary canal in lampreys. That the intestinal portion reacts to treatment with Ach is in good agreement with the fact that the innervation in this part is exclusively from the vagus. In the rectal portion an obvious effect was observed with Ach and a strong effect with 5-HT. This is interesting in combination with the spinal innervation which is peculiar to the rectal portion of the alimentary canal.

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ON THE PERIPHERAL NERVOUS SYSTEM OF ASCIDIANS

BY

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The aim of the present investigation is to give a brief account of some anatomical and embryological features in the nervous system of ascidians as this subject seems previously to have been little investigated and the results may be of some value to further studies on related problems.

The work was mainly carried out at Kristinebergs Zoologiska Station, Fiskebäckskil, but part of it was fullfilled at Stazione Zoologica, Naples. This gives the author the pleasure of expressing his most cordial thanks to the directors, Dr. G. GUSTAFSON, Kristineberg, and Dr. P. DOHRN, Naples, for faciliating the work at these stations in every respect.

For financial support the author is indepted to Liljevalch Jr:'s Resestipendiefond and to the Swedish Natural Science Research Council.

MATERIAL AND METHODS

Most of the present observations were obtained by phase contrast microscopy on living Ciona intestinalis (L.) from immediately after metamorphosis to the age of about one month. In the stages in question, the intravital methylene blue technique of Unna as modified by C. H. MCCONNEL (2) and J. E. SMITH (9) often proved valuable. Concerning sensory structures most data were obtained by vital staining of full-grown Ciona with methylene blue. Even though varying greatly for different materials, close conditions were important for a satisfactory result by this staining. A solution of in sea water saturated polychrome methylene blue diluted five times and made neutral or slightly acid gave the best results. For some tissues the staining time was about 10 hours, for others 20 minutes were sufficient. Low temperature proved to be very favourable for staining in most cases. In some cases the best results were obtained by keeping

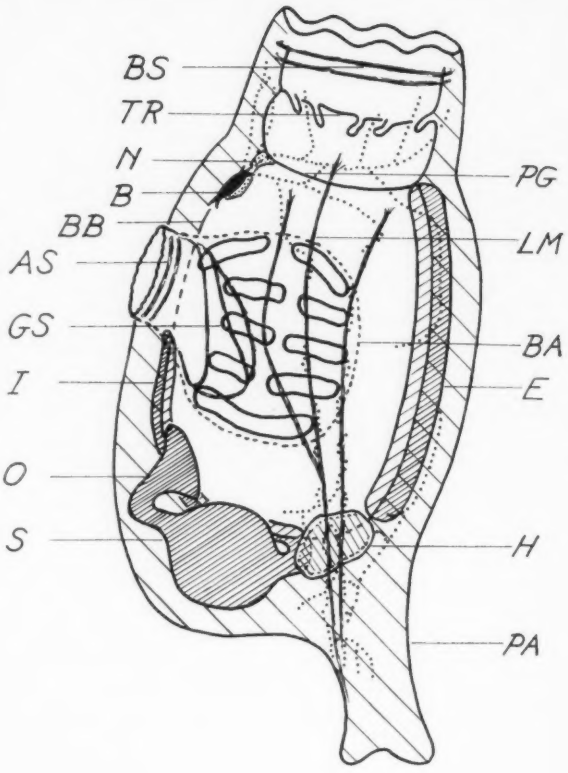

Fig. 1. An about 3 mm long Ciona intestinalis seen from the right side. The mantle is not drawn in this and the following drawings. *AS* Atrial Sphincter, *B* Brain, *BA* Border of Atrial Room, *BB* Border of Branchial Gut, *BS* Branchial Sphincter, *E* Endostyle, *GS* Gill Slit, *H* Heart, *I* Intestine, *LM* Longitudinal Muscle, *N* Neural Gland, *O* Oesophagus, *Pa* "Postabdomen", *PG* Peribranchial Groove, *S* Stomach, *TR* Tentacule Ring.

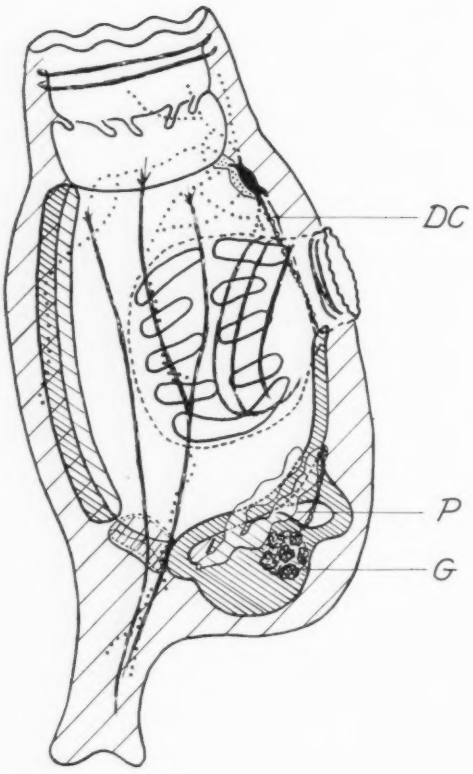

Fig. 2. The same animal as in Fig. 1 seen from the left side. *DC* Dorsal Cord, *G* Gonad, *P* Pyloric Gland.

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the tissue—fastened with needles on a paraffine plate—hardly more than moist by a gently streaming dye solution.

OBSERVATIONS

The author has not observed any peripheral nerves in the ascidian tadpole. After metamorphosis, however, nerves appear in ever increasing number and size. Gradually great individual variation and many anastomoses become apparent (Fig. 1 and 3). Later on, however, the pattern of the peripheral nerves grows more uniform apart from the bilateral asymetry which is characteristic of Ciona and other ascidians studied in this respect (Fig. 1 and 2).

Nerve cell bodies were never observed outside the brain. In the nerve axons phase contrast microscopy revealed smaller irregularities in form and structure which might be of some interest from the physiological aspect.

The nerves mainly leave the brain from its anterior and posterior ends where they are concentrated into rather thick roots.

Anterior brain nerves innervate the branchial siphon and most of the body wall. The branchial siphon seems to be the best innervated part of the ascidian, and especially in the branchial tentacules (Fig. 3 b)—as well as in the border from which they emerge—nerves form dense "plexuses" with many free nerve endings. Other sensory structures observed in connection with nerves are the pigment spots in the border of the siphon (see P. Brien (1) for references), pear-formed structures with bristles found in the surface of the tentacules (Fig. 3 b), and spindle-shaped sensory cells of the body wall (Fig. 6 a).

The ring muscles in the branchial siphon and most of the longitudinal muscles receive anterior brain nerves and are provided with characteristic nerve endings (Fig. 4). Sometimes it could be established that one and the same muscle was innervated more than once along its course and from different brain nerves.

Some nerves follow longitudinal muscles closely (Fig. 1 and 2). In the abdomen they give off fibres to the body wall. Mainly, however, they form a "plexus", together with other nerves, in the postabdomen of the young Ciona. They are also observed in its residue in full-grown animals and seem to innervate the mantle where it was possible to observe nerves.

Near the posterior end of the endostyle on the left side a nerve was observed that gives off some branches to the stalk. Other of its fibres, however, could be traced to the area near the hypobranchial end of the pericardium. This nerve comes from one of the right anterior roots of the brain, and thus it describes a big half spiral rather superficially in the bodywall (Fig. 1 and 2).

Posterior brain nerves innervate the atrial room and the atrial siphon (Fig. 4), parts which are rather poorly innervated compared with the branchial siphon.

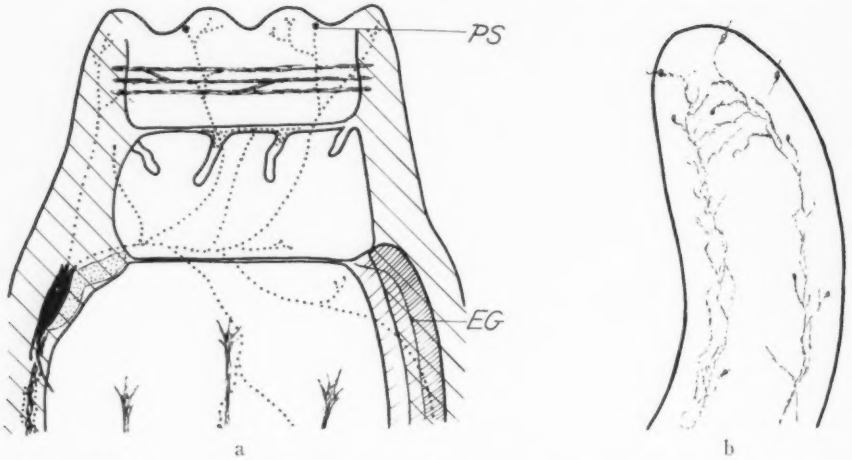

Fig. 3 a. The right side of the branchial siphon of a Ciona of about the same size as in Fig. 1. *EG* Bottom of Endostyle Grove, *PS* Pigment Spot.

Fig. 3 b. A branchial tentacule with nerves and sensory structures.

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There are evidently certain complications concerning the ring muscles and the innervation at the fusion of the pair of atrial siphons in the young animal. Some longitudinal muscles are innervated from posterior brain nerves. This investigation revealed no sensory structures but free nerve endings in these parts.

All the gill slits are well innervated from a pair of deeper lying nerves emerging from the posterior brain roots (Fig. 4). An innervation of the gill slits was to be expected because of their correlated activity. Thus the regularly occurring interruptions in the ciliar movements are simultanous for all slits of both sides of the branchial gut. Among other irregularities in the ciliar activity weak dilutions of alcohol or ether block correlation of interruptions between the two sides, while somewhat stronger solutions make all the gill slits work irrespective of each other.

The dorsal cord runs between the neural gland and the gonads and is a prominent structure of the young ascidian. In the discussion of the origin and nature of the dorsal cord, M. FEDELE (4 and 6) asserts that it belongs to a visceral nervous system. From the right posterior brain root some tiny nerves emerge evidently the same as that referred to by J. HUUS (7) as the dorsal nerve. The nerves go to the dorsal cord and follow it through its whole length, intimately surrounding it in a plexus-like manner (Fig. 3 and 5). From here the abdomen is innervated as well as the posterior part of the branchial gut and the endostyle.

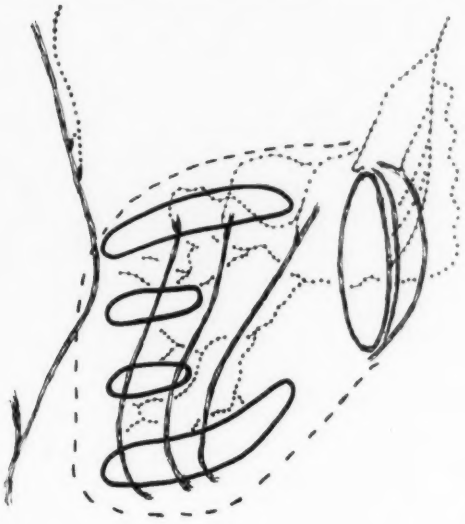

Fig. 4. The left atrial room of an about 2 mm long Ciona. The atrial opening is not drawn.

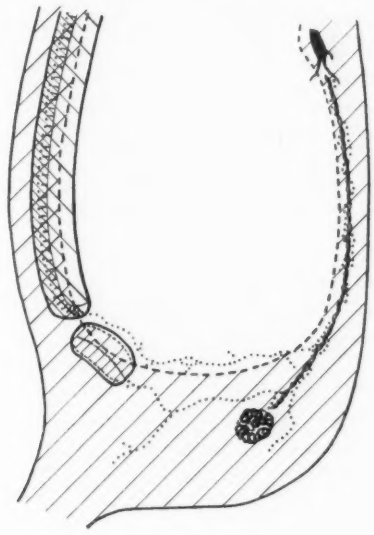

Fig. 5. The "dorsal cord plexus". The only other organs outlined is the brain, the dorsal cord, the gonads, the heart and the endostyle with its sensory cells.

Several nerve fibres are lost out of sight among the gonads, which in the young Ciona form a loose coalescence of smaller parts. These could be seen performing rythmical movements of attraction and repulsion to each other.

As was the case with one of the anterior brain nerves, fibres emanating from this "dorsal cord plexus" could be traced to the proximity of the pericardium, but this time its visceral end. In the suture between the heart and the pericardium of the adult Ciona elongated structures were observed which the author presumes to be sensory cells (fig. 6 c). They occur after methylene blue staining of dissected hearts and are rather evenly scattered along the whole suture. Fibres could be seen leaving them, but the further connection has not yet been established. The author will not ascribe these structures a significance for the conduction of peristaltic waves as the cutting through of the suture does not stop the waves; this in contrast with the results obtained when the rest of the heart

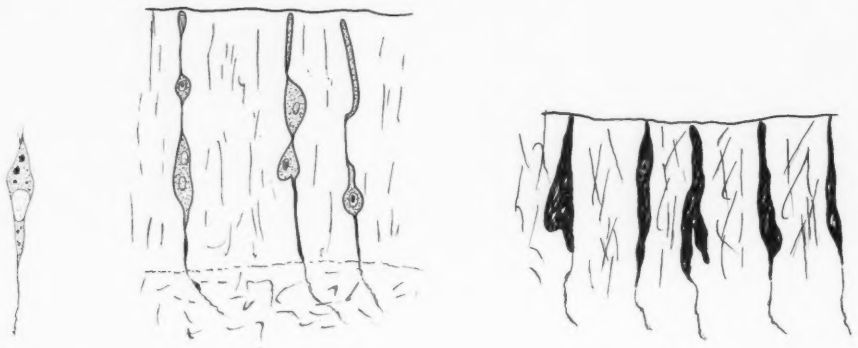

Fig. 6. Sensory cells: a) from the body wall; b) from the endostyle; c) from the heart. Magnification about 500 times.

sac is cut through. After that these experiments were made the author learns through B. J. KRIJGSMAN (8) that also A. EBARA has observed possible nervous elements in this suture though apparently of different appearence. More interesting is that EBARA comes to the opposite results when damaging the suture and the rest of the heart sac.

Concerning the intestinal tract, the author has not yet ascertained nervous elements in it. The endostyle, however, is rather well innervated by nerves running along the bottom of the branchial sac and entering the endostyle in its posterior end (Fig. 5). Fibres could be seen terminating in elongated sensory cells (Fig. 6 b) in the bottom of the endostyle groove.

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THE MORPHOLOGICAL AND CHEMICAL CHARACTERISTICS OF ORGANIC CRYSTALS IN THE REGENERATING SHELL OF HELIX POMATIA L

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In the Roman snail, like in all other molluscs, the development of the shell proceeds gradually, as the formation of an organic membrane, matrix, and a subsequent mineralization of this membrane which results in the deposition of calcium carbonate crystals in it. Hitherto it is not fully understood, whether during the mineralization $CaCO_3$ is immediately deposited in the matrix, as is supposed by Moynier de Villepoix (1892), de Waele (1930) Robertson (1941), Wagge (1951), Heaysman (1951), and others, or an initial desposition takes place of calcium phosphate which is subsequently transformed into $CaCO_3$, as Biedermann (1902), Manigault (1939), Bevelander and Benzer (1948), Bevelander (1952), and some other authors believe.

Some early authors, and recently de Waele (1930) and Manigault (1939), considered mineralization to be induced by some complex organic substance. This substance appears in the pallial fluid, and is transported to the place of shell formation. Thus de Waele assumed that in Anodonta calcification begins with the appearance of a protein-calcium-carbonate complex, whereas Manigault supposed that in the Roman snail the presence of a "calcaffine substance" is needed for this purpose. By use of muci-carmine and toluidine blue stains Manigault showed that this "calcaffine substance" might be a chondroprotein or a mucoprotein similar to a substance of this kind found in the cartilage of vertebrates.

The completest description of the morphology of the calcifying elements of the shell of Helix pomatia is given by BIEDERMANN (1902). This author has described the formation of the shell during the embryonic development, in growing animals, and after damages to the shell. According to BIEDERMANN the calcification of the matrix proceeds in different ways in embryos and in growing animals, on one side, and in the regenerates, on the other side.

According to BIEDERMANN the process of shell development is easily observed in embryos shortly before hatching. Calcification beginns with formation of roundish and rosette-shaped calcifying plates. These plates show a bright and compact centre and a porous periphery, various elements with different ability of optical refraction being joined together. Not far away from the outer margin of the newly formed calcareous layer the plates combine together, and form a continuous sheet of polygonal flattened corpuscles. Still further away from the outer boundary the structure of the calcifying membrane is more complicated. The polygonal plates were transformed into a kind of fine calcareous fibres, and the normal shell elaboration is attained. According to BIEDERMANN the round plates are not birefringent, and are composed of dicalcium phosphate. They become birefringent only upon the deposition of the carbonate in the plates. On this account birefringence can be used as an index for the detection of calcium carbonate in the crystals.

Also in the growing shell of adult snail the mineralization is characterized by the deposition of small round plates. They are weakly birefringent, but on growing larger their central parts soon show quite strong birefringence. After some time the round plates transform into "warts" with granular structure. This form of calcifying elements was not observed in the embryonic shell formation. Even the "warts" show stronger birefringence in the centre, and have very irregular boundaries. The layer of the "warts" grows until a thick calcareous sheet has been formed.

A somewhat different account is given by BIEDERMANN of the calcification in regenerates from older shells. Upon removal of a small piece of the shell the wound is closed already after 1—2 hours by a thin membrane impregnated with some calcareous corpuscles. The corpuscles have different size and form, but can on the whole be characterized as spherulites. They are composed mostly of $CaCO_3$, and in polarized light show strong birefringence.

Similar is the process of regeneration of the newly formed shell, after the resection of a small portion, or if the whole newly formed part of the shell is removed. In this case, as in the foregoing, irregular small or very small spherulites of calcium-carbonate are deposited in the organic membrane. Sometimes they are completely round, and in polarized light show a dark cross and rings of interference colours. With the increase of the number of spherulites they begin to form a continuous layer. The mutual pressure imparts to the corpuscles a polygonal shape. The layer of the spherulites in the regenerating shell, according to BIEDERMANN, is not analogous to the corresponding part of the normally developing shell. The author considers the spherulite layer as some kind of pathological product of emergency under protection of which the new inner part of the shell will be developed. The difference between the normally developed and the regenerated shell is expressed by the non-equivalence of the mantle zones producing the shell. The normally formed shell can be produced only by the epithelium of the mantle edge. The epithelial cells of all other parts of the mantle can form only irregular calcareous layers, composed of spherulites.

The aim of this investigation was to analyse in greater detail the early mineralization stage of the regenerating shell of Helix pomatia, with special regard to the morphology and chemistry of the calcifying elements. The damaged shell

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of the snail is quickly repaired, yet, slowly enough to afford a good opportunity for the observation of the different stages of the regeneration.

MATERIAL AND METHODS

Adult animals were used, weighing about 20—25 g. Small pieces of shell, about 25 mm², were removed at a distance of 3—4 cm from the peristome of the shell. The regenerating membranes (together with a portion of surrounding old shell, were collected from animals which were decapitated at different times (6—24 hrs) after the shell damage. The membranes were fixed in neutral buffered formaldehyde solution of pH 7.0 (LILLIE, 1954), or in cold absolute alcohol with 5 % glacial acetic acid (KONING and HAMILTON, 1951). All fixed regenerates, such without further treatment as well as those treated by different histochemical reactions, were mounted in Permount. For comparison unfixed regenerates were examined. The formation of crystals was examined in the ordinary microscope, in the phase contrast microscope, and in polarized light. Yet, the results in polarized light are given here with reservations for the properties of intrinsic birefringence which will be discussed in another paper. The following histochemical reactions were applied to regenerates. For the detection of proteins: the methods of MAZIA, BREWER and ALFERT (1953), and of GRAM—WEIGERT (1903; LILLIE, 1954); for nucleic acids—methylgreen pyronin Y stain (TAFT, 1951; KURNICK, 1955); for metachromatic reaction—toluidine blue stain, 0.1 % solution in distilled water, or 0.05 % solution in citrate buffer of pH 4.5 (LILLIE 1954; ATKINSON, 1952). Polysaccharides, neutral and acid mucopolysaccharides, and mucoproteins were localized by periodic acid-Schiff (PAS) reagent (LILLIE, 1954; ATKINSON, 1952; GLEGG, CLERMONT, and LEBLOND, 1952). Calcium was identified by the method of DAHL (1952) with alizarine red S. The enzymes used were: crystalline ribonuclease (Nutritional Biochemicals Corporation, Cleveland, Ohio) in the concentration of 0.25 mg/ml in distilled water, for 2—4 hrs at 57° C.; testicular hyaluronidase (Hyalas, A/B Leo, Hälsingborg) in the concentration of 100 V.R.E./ml in 0.7 % NaCl, for 18 hrs at 37° C.; trypsin in the concentration of 1 % in phosphate buffer of pH 7.59, for 3 hrs at 37° C.; diastase (Merck, Darmstadt) in the concentration of 1 % in phosphate buffer of pH 6.0, for 3 hrs at 37° C. The extraction of lipids was carried out with hot methanol-chloroform (GERSH, 1949) for 18 hrs, at 60° C.

RESULTS

a) *Morphology and development of organic crystals in the regenerating membrane.*

As mentioned before, the repair of the damaged shell is accomplished in quite a short time. Already after 1—2 hours the place of damage is covered by a thin organic sheet. After 24 hours the regenerating membrane is so far calcified, and the calcium carbonate crystals lie so close to each other, sometimes in two or three layers, that the observation under the microscope gives no account of their formation. The youngest regenerating stages are most suitable for the study of the mineralization of the organic membrane. Particularly the regenerates of 6—12 hours show an instructive arrangement of calcifying elements. If the regeneration proceeds regularly, without the participation of remnant splinters of the old shell, the early stages of the mineralization, viz. minute initial granules and round or elongate, boat-like, disks, prevail in the periphery of the regenerating membrane. Towards the centre disks with several concentric or elliptic rings, spherulites, and conjugated pairs of spherulites adjoin to these elements. The added figures show the general types of observed calcifying bodies. For the data of their dimensions see the explanations of the figures.

In our preparations (Fig. 1, 2, 6) the minute granules did not always show birefringence. But as soon as they become larger, flattened disk-like, and differentiate into central and peripheral parts, they begin to exhibit birefringence. Between crossed Nicols the round disks appear bright, with a dark central granule. On insertion into the polarizing microscope of the first order red retardation plate, and focusing a round disk so that the central granule is sharply adjusted, the entire peripheral surface of the corpuscle shows an uniform interference colour of the second order blue or of the first order yellow. On rotation of the preparation through about 90° the blue disks change their colour into yellow and, contrariwise, the yellow—into blue. The dark cross of Brewster is not present. To judge from these observations, the round disks show a linear polarization in only one direction, notwithstanding the circular form of the disks themselves. When the disk itself has a blue colour, the central granule within it appears in the background colour first order red. The central granule of a yellow disk appears orange-red. Obviously, the centre of the round disk possesses only a very feeble birefringence. It appears indeed dark between crossed Nicols without retardation plate, as already mentioned. Between crossed Nicols the border of the disk is seen as a black line. On insertion of the first order red retardation plate the addition colour of the border of the disk still remains black, but the subtraction colour changes into orange-red. From this it

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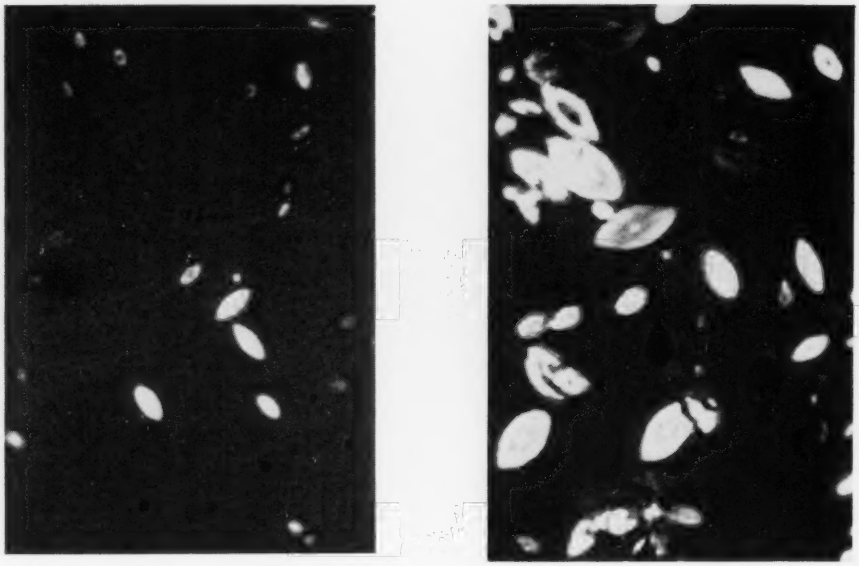
can be concluded that the surface of the round disks has a still weaker birefringence than the central granule.

The round disk continues to grow and deposits a new layer of substance upon its surface, but still lacks the Brewster's cross. Between the crossed Nicols such a disk, like the previously mentioned, is bright with black border and dark central part. When the first order red retardation plate is inserted, in addition position the centre appears red, then follows the blue middle part, limited by a fine dark line, a narrow first order red coloured peripheral part, and the black border. The peripheral part which shows a weaker birefringence is obviously the lastly depostited substance (Fig. 3). The next stage of the development of the round disks seems to be a plate in which the sharp delimitation of interference colours upon the surface has disappeared. The addition colours of the disk now change very gradually from the periphery towards the centre from blue to green and yellow. In subtraction position almost the entire surface of the disk appears faint yellow with greyish reflexes towards the centre. The Brewster's cross in still absent. The described character of the interference colours shows that, during this stage, some rearrangement of the elements producing the polarization takes place within the disk. The arrangement of structural elements, which previously was probably linear, apparently gives way to a more equal state with a gradual change of birefringence along the radii towards the centre (Fig. 5). It is possible that simultaneously also some change of the intrinsic birefringence of substances of the disk is going on. Yet, as already mentioned, all relations between the form and the intrinsic birefringence of the calcifying shell elements will be discussed in another paper.

Further growth of the round disk can result in the formation of a very regular body resembling the spherulite. Such a body shows an incomplete Brewster's cross. However, usually the round disk distorts and transforms into a multilobular body (Fig. 6). This process is accompanied by new changes of the birefringence within the disk, as different interference colours become irregulary distributed over the whole surface of the corpuscle. In the last stage of the development both kinds of the round disks transform into more or less regular clusters of radially outgrowing needles (dendrite-like spherulites). Obviously this process of the spatial separation within the disk was induced by the structural differentiations observed during the previous stage of the development.

A second type of the calcifying elements are the typical spherulites. They are larger than the round disks, and in fully developed condition show very distinct concentric stratification. They originate from broad elongated corpuscles with strong negative birefringence on either end. Additional substance is deposited on the sides, and a short-armed cross is formed. Sometimes such a cross is composed of four identically shaped corpuscles (Fig. 9). Further growth results in the fusion of the arms of the cross and the formation of the



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EXPLANATION OF FIGURES

Photomicrographs of organic crystals in regenerating shell membranes of Helix pomatia as observed 12 hours after the shell damage. Fixation: neutral buffered formaldehyde.

Fig. 1—4. Early stages of the development of round and boat-like disks. Polarizing microscope with first order red retardation plate.

Fig. 1. Fig. 2.

Fig. 1. The minute round granules appear dark grey, they are not birefringent. The larger, black ones, are in the position of extinction. Diameter — 0.6 μ—1.0 μ. At the top of the photomicrograph some stages of further development of round disks: birefringent bodies with dark central part. Diameter — 1.2 μ—2.0 μ. Below—a few birefringent boat-like disks of different size. Length —3.0 μ—5.5 μ. Magnification × 1100.

Fig. 2. Round and boat-like disks from a later stage of the development, all being birefringent. In the centre—round disks, some in the position of extinction. Diameter — 4.0 μ—5.5 μ. Diversely oriented boat-like disks. Length — 5.0 μ—11.0 μ. Magnification × 1100.

spherulite. Between crossed Nicols the spherulite appears bright greyish with black Brewster's cross. With the first order red retardation plate inserted a spherulite with 1—3 rings exhibits all over the surface the second order blue in negative sectors and the first order yellow in positive sectors (Fig. 10). Brewster's cross consequently is negative. Since the structure elements of spherulite are arranged tangentially, the longer axes of their index ellipsoides must be positive. The spherulite with four rings shows, like the foregoing one, a negative Brewster's cross, but now a series of interference colours appear in each ring (Fig. 11). In negative quadrants the colours within each ring change from

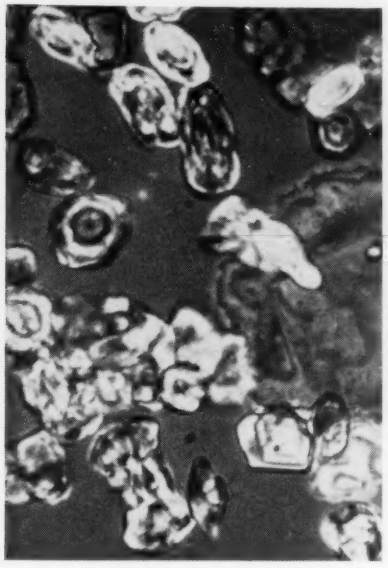

Fig. 3.

Fig. 4.

Fig. 3. Round disks with two sharply limited birefringent rings, dark centre and dark boundaries. Diameter — 8.0 μ—12.0 μ. Boat-like disks, some with started lobulation. Length — 15.0 μ—18.0 μ. Magnification × 700.

Fig. 4. Two large boat-like disks, one in the addition, another in the subtraction position. Length — 12.0 μ—13.5 μ. Observe the more or less regular interference rings within the disks. Magnification × 1100.

the centre towards the periphery in the sense of increasing birefringence within the second order colour scale (from blue, through green and yellow, to red). The rings are separated by thin black zones without or with very low birefringence. In positive quadrants (subtraction position) the value of the interference colours decreases, as expected, in each ring from the centre towards the periphery from pale yellow of the first order to black. It is not yet possible to decide whether the complication of interference pattern is due to an increasing differentiation of spherulite substance (structure) or only to a continual apposition of new substance in every ring. The spherulites with four rings often show interference colours even without the retardation plate in the path of the light. Analogous statements can be made about still bigger spherulites with 5—7 or more rings. Also here the mentioned succession of the interference colours can be observed within each ring. Due to the spatial separation along their radii, in the last sequence also the spherulites transform into the clusters of needles—dendrite-like spherulites (Fig. 12, 15).

The third type of calcifying corpuscles are the elongated, boat-like disks (Fig. 1, 2). These seem to originate from two initial granules which are se-

Fig. 5—8. Advanced stages of the development of round and boat-like disks. Ordinary light microscope. Interference filter of peak wave-length 547 m μ. Magnification $\times$ 700.

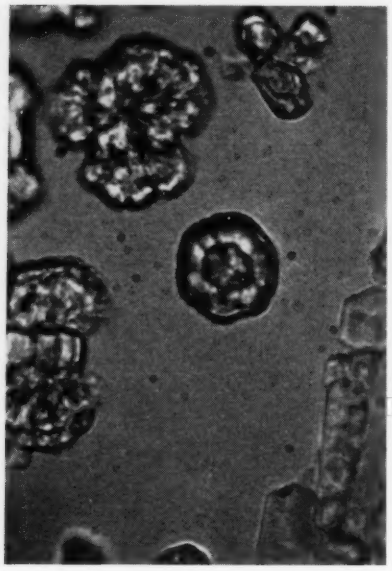

Fig. 5.

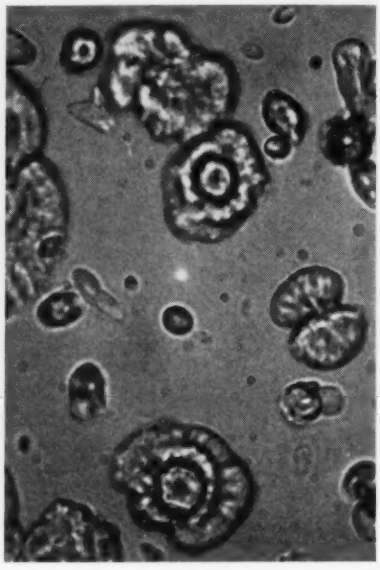

Fig. 6.

Fig. 5. In the centre a round-shaped disk consisting of two sharply limited rings. The central granule very small, the border dense. The beginning of the radial differentation. Diameter — about 17.5 μ. Some lobulated boat-like disks.

Fig. 6. Two round disks in a state of irregular radial lobulation. Size — 17.5 μ—22.5 μ and 20.0 μ—23.5 μ. At the right, a paired spherulite-like body. Observe also some early stages of round disks.

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condarily enclosed in common elliptical sheets of calcifying substance. Between crossed Nicols the youngest of them appear bright with a black boundary and a dark central part. On insertion into the path of the light of the first order red retardation plate they exhibit a negative birefringence along their length, i.e. they are blue when placed with the long axis parallell to the a-axis of the retardation plate, and yellow when placed perpendicular to the a-axis. The central part of such a boat-like disk has the addition colour first order red, the subtraction colour being first order reddish-orange. The contours of the blue-coloured plates are black, those of the yellow-coloured reddish-orange. Consequently, the boat-like disks, similar to the round disks, have only a feebly birefringent central part and a boundary with a very weak or without any birefringence. In the next stage of development the surface of the boat-like disk exhibits a series of interference colours instead of one uniform colour, as before (Fig. 4). The addition colours change within the second order from

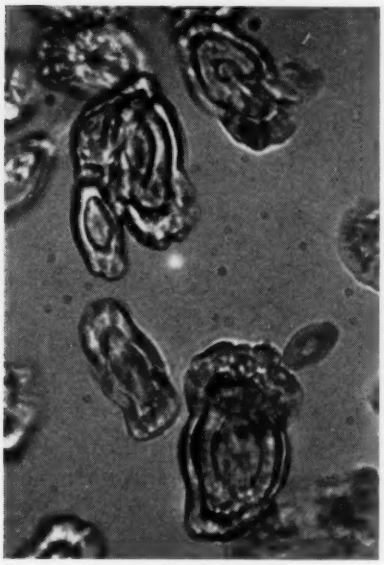

Fig. 7.

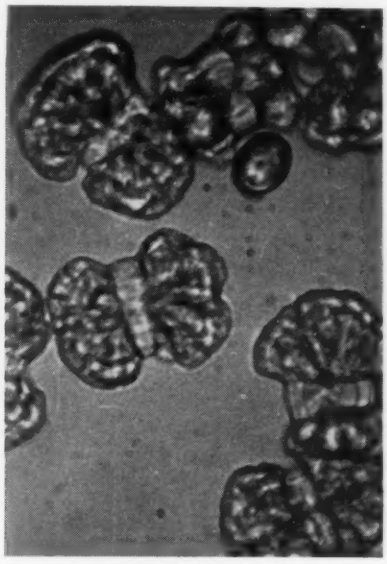

Fig. 8.

Fig. 7. Boat-like disks with alternating light and dark elliptical rings. Observe the beginning of the lobulation at the ends of the disks. The length of the largest disks — 25.0 μ—27.0 μ.

Fig. 8. Boat-like disks in the stage of fully lobulated end regions. Length — 28.0 μ—30.0 μ.

blue to green and yellow towards the centre and parallel to the longitudinal axis, i.e. increasingly from the periphery towards the centre. This obviously indicates the initiation of some structural differentiation within the disk due to its growth.

In the course of continued growth the boat-like disks change their character of birefringence still further. They begin already to show interference colours between crossed Nicols only: bright light-yellow with orange-brown centre. Inserting the retardation plate, and orientating the corpuscles with their longitudinal axes parallel to the *a*-axis of the retardation plate, we now observe the black boundary, the yellow-orange peripheral zone, and towards the centre, first a red then a green or blue zone. With the longitudinal axes at right angles the same plates show an orange-red border and a black central portion, whereas the prevailing part of the surface exhibits a straw-yellow colour with a grey tinge. I am still unable to say whether the last described change of interference pattern indicates only some further rearrangement of structural elements, or perhaps even some inversion of birefringent qualities of these elements themselves. A further study of the relation between the intrinsic and the form birefringence is needed also in this case.

Fig. 9—12. Different stages of the development of spherulites. Polarizing microscope.

Fig. 9.

Fig. 10.

Fig. 9. The earliest stages of the spherulite: an elongated body with birefringent ends, about 5.0 μ—5.5 μ in the length. Other bodies showing formation of the short-armed cross. Diameter — 4.5 μ—5.0 μ. Magnification × 1100.

Fig. 10. Spherulites with 1—3 concentric rings and black Brewster's cross. Diameter — 12.5 μ—13.7 μ. Magnification × 1100.

The next stage in the development of the boat-like disks is that in which the regular orientation of interference colours disappears. The central (red, green, and blue coloured) zones of the disk are the first to be involved in this disarrangement. The boat-like disk lengthens. At the ends of the disk appear simultaneously transversal series of interference colours which resemble those already described within the rings of the large spherulites. In continuation the ends of the boat-like disk begin to grow faster than the central part, get wide and lobulated, develop fan-like and spread out towards both sides (Fig. 7, 8). At the same time the polarization pattern at the ends of such disks undergoes some desorientation, and spreads in different directions. By further extension and differentiation of the terminal parts and by shortening of the central part of the disk a typical paired needle cluster—a couple of dendrite-like spherulites—(Fig. 13, 14) is developed. As follows from previous, all kinds of calcifying elements finally produce more or less regular needle clusters which in their turn yield the definitive polygonal crystals of calcium carbonate.

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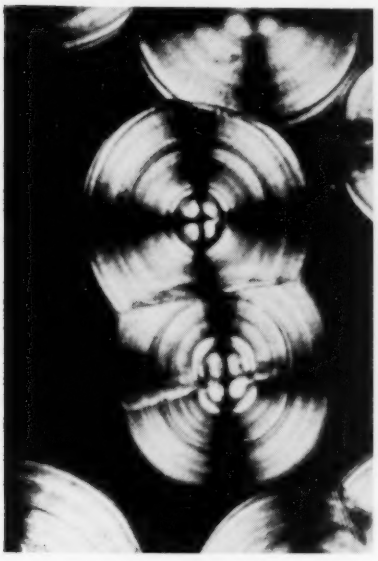

Fig. 11.

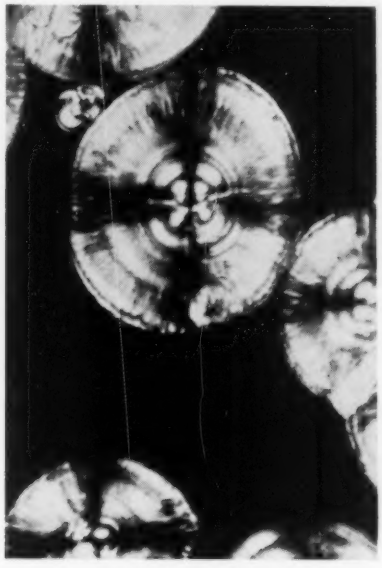

Fig. 12.

Fig. 11. Spherulites with many concentric rings and black Brewster's cross. Diameter — 35.0 μ—37.0 μ. Magnification × 700.

Fig. 12. The beginning of the formation of radially arranged needles within the spherulite. Diameter — about 42.0 μ. Magnification × 700.

Apart from the aggregations of the described regularly developed disks, also much more irregular combinations of triple and quadruple spherulites and clusters occur. The occurrence of the last-named forms was observed especially when the number of calcifying elements was very large, and the free space for their growth limited. Apparently the size and differentiation stage at which every calcifying corpuscle can arrive before aggregating with another corpuscle is largely influenced by the extent of the existent free growing space.

It is now of great interest that the just described different forms of corpuscles (disks and spherulites) from the early stage of the mineralization are identical with the organic crystals which are formed by all native and synthetic polymers, as described recently by BRENSCHEDE, W., JENCKEL, E., MÜNSTER, A. und STUART, H. A. (1955) and RÅNBY (1957). We can therefore make our description more definite by saying that the mineralization of the shell in Helix commences with the formation of polymer organic crystals.

b) *The chemical composition of the organic crystals.*

In order to discover the chemical nature of the organic crystals of the early mineralization stage, a number of histochemical tests was performed using the regenerated membranes. The following results have been obtained.

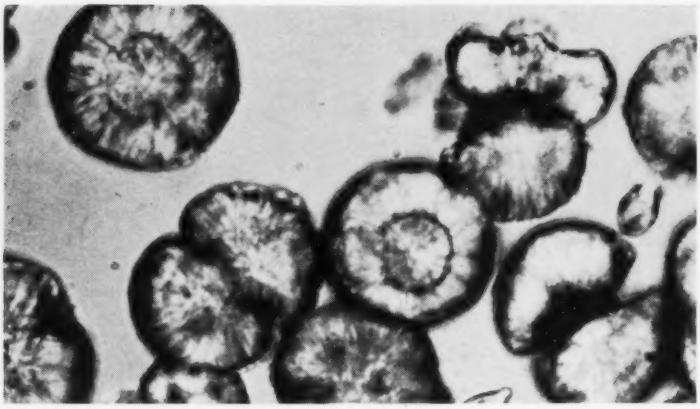

Fig. 13. Round and boat-like disks starting the development of singular and coupled spherulite-resembling bodies with radially arranged needles. Ordinary light microscope, interference filter of peak wave-length 547 m μ. Diameter of the round disks 28.0 μ—30.0 μ; the length of the boat-like disks about 34.0 μ. Magnification × 700.

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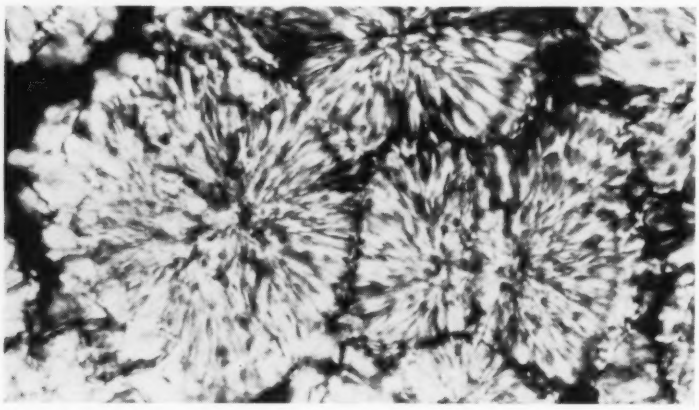

Fig. 14. Needle-clusters—dendrite-like spherulites—derived from singular and coupled spherulite-resembling bodies. Diameters — 50.0 μ—55.0 μ. Polarizing microscope. Magnification × 700.

At first, the periodic acid-Schiff reaction (PAS) was performed in the regenerating Helix shell membranes. The PAS reaction must be considered as quite unspecific. Positive results are given by all substances which contain 1,2-glycol or α-amino alcohol groups, and after periodic acid oxidization produce aldehyde groups which react with Schiff's reagent (GLEGG, CLERMONT, and LEBLOND, 1952). Such substances are polysaccharides, acid and neutral mucopolysaccharides, mucoproteins, and, theoretically, some proteins. More-

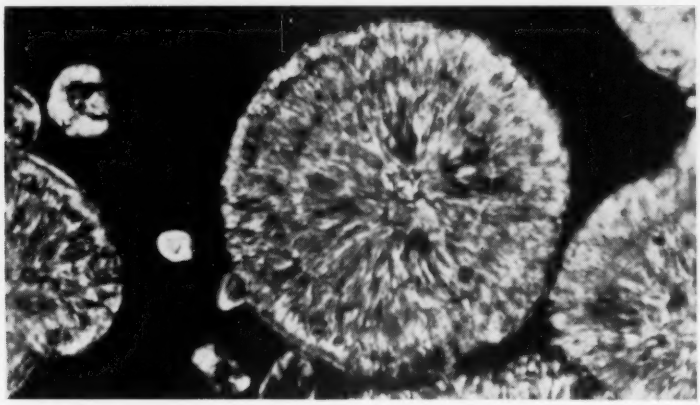

Fig. 15. Fully developed needle-cluster—dendrite-like spherulite—from a typical spherulite. Diameter — 55.0 μ. Polarizing microscope. Magnification × 700.

over, the Schiff's reagent contains hydrochloric acid in quite large quantity, and not only the organic crystals, but also the definite calcium-carbonate crystals of the shell become for the greatest part destroyed. In remnants of crystals the membranes surrounding both kinds of them proved to be PAS positive. Positive are also the central parts of organic crystals. This result indicates that in the membranes and also in the organic crystals themselves there must exist a substance or substances belonging to the above mentioned three groups. For a more precise identification of the kind of the substance(-es) further histochemical tests were carried out.

Methylgreen pyronin Y stains the crystals red, and the colouration is not removed by ribonuclease. The organic crystals are not destroyed and they lose not the birefringence following to the application of the methylgreen pyronin dyes nor after the enzymic hydrolysis of the ribonuclease. Very significative results were obtained by staining with toluidine blue in citrate buffer pH 4.5 or in distilled water. The organic crystals became disrupted both in aqueous and in buffered dye solutions, and lost their birefringence. They lost calcium upon the action of both the citrate buffer and the dye itself. As to the lastly mentioned effect, the result can be satisfactorily interpreted if we admit the presence of polysaccharide sulfate esters in the crystals. MILLER, WALDMAN, and MCLEAN (1952) found that toluidine blue and other basic dyes, which have a high affinity for polysaccharide sulfate esters, can prevent the calcification of cartilage in vitro. It seems therefore that toluidine blue is bound to the polysaccharide sulfate ester molecule by the same reactive groups as Ca. In our case the toluidine blue is stronger in the competition for the reactive groups than the calcium bound to the organic crystals. Calcium is thrown out, and the crystals become partly destroyed. The residues of such crystals show

metachromatic reaction. The only exception is the outer boundary which often is not metachromatic, and evidently composed of small granules. The metachromatic staining remained after action of ribonuclease. Metachromasia was observed even after the extraction of lipids with hot methanol and chloroform. All mentioned positive tests suggest that the substrate of the organic crystals in basophilic. SCHUBERT and HAMERMAN (1956) confirmed that metachromasia reveals substances of high molecular weight with free anionic groups. These substances include anionic mucopolysaccharides, nucleic acids, and some anionic lipids. However, in our preparations metachromasia was not destroyed by ribonuclease nor after the extraction of lipids, and there occurred no positive methylgreen reaction for deoxyribonucleic acid. Thus, the results suggest that the organic crystals contain some acid mucopolysaccharide. Also the results of the pyronin Y-test suggest a similar conclusion. Pyronin Y gives positive reaction only with ribonucleic acid, depolymerized desoxyribonucleic acid, osteoid, and eosinophilic granules (KURNICK, 1955). A positive result of the pyronin Y-test, supplied after RNase-hydrolysis, indicates therefore that the organic crystals contain a substance similar to the osteoid, a calcifiable ground substance in the mineralizing tissues of vertebrates.

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Further support for the assumption that in the organic crystals of the shell of the Roman snail calcium is bound by some acid mucopolysaccharide (chondroitin sulfate) can be derived from a paper by EINBINDER and SCHUBERT (1951). These authors obtained pure calcium chondroitin sulfate crystals from the cartilage of the bovine trachea and nasal septa. These crystals have the same form as the previously described end stages of the calcifying elements from the regeneration shell of Helix. But as already mentioned, RÅNBY (1957) has recently established, that all organic polymers can give similar crystalline forms. In order to be quite sure that the polymeric substance in the organic crystals of the regenerating shell is really an acid mucopolysaccharide (chondroitin sulfate) we have submitted the membranes to the action of testicular hyaluronidase. According to MEYER and RAPPORT (1951) testicular hyaluronidase hydrolyses some fractions (A and C) of chondroitin sulfate. LILLIE, EMMART, and LASKEY (1951) have demonstrated that bovine testicular hyaluronidase can destroy all of the metachromatically staining materials of umbilical cord matrix and arteries, and of the ground substance of cartilage. In our experiments with the testicular hyaluronidase various organic crystals were only partly destroyed. They also partly lost the birefringence, and the boundaries became less sharp (? hydration of some substance in the crystals). When, however, trypsin and hyaluronidase were applied in succession, the organic crystals were completely destroyed. After successive action of both enzymes the remnant of the crystals took up no more pyronin Y. Only in few cases the centre of the calcifying element still appeared red, but also then the birefringence was lost.

KUNKEL and TISELIUS (1951), and GESCHWIND and LI (1952) introduced

bromphenol blue for the identification of amino acids, as well as proteins in general, in the electrophoresis and paper chromatography. MAZIA, BREWER, and ALFERT (1953) first adapted this stain for the identification of the above mentioned substances in tissue sections. The method is delicate in contrast to other histochemical methods for protein identification which operate with strong reagents deforming tissues. Following upon the application of bromphenol blue all organic crystals of the shell membrane showed positive reaction for proteins. However, the crystals lost the birefringence. In addition, the staining according to the GRAM—WEIGERT method indicated that the protein in the organic crystals must be collagen or a collagen-like substance. Following to the application of the GRAM—WEIGERT stain the crystals not only lost the birefringence but also their inner structure. The cause of the loss of the birefringence following to the application of bromphenol blue and GRAM—WEIGERT was, presumably, the breakdown of bindings between the calcium and the organic substrate within the crystals. Also an intrinsic alteration of the birefringence of the substrate itself is not excluded.

The last two positive reactions together with the establishment that hyaluronidase and trypsin, applied simultaneously, completely destroy organic crystals, verify that in our organic crystals an acid mucopolysaccharide (chondroitin sulfate) is combined in some manner with a protein (collagen). Whether protein and acid polysaccharide occur in crystals only in the form of a complex, or can exist there independently, or combined with some other substances, further experiments must show.

The question about the kind of chemical bindings in which calcium occurs in the crystals of the regenerating membrane lies beyond the scope of this investigation. This problem will be discussed in another paper. Yet, already here one comment might be made on this question. All organic crystals exhibit positive reaction for calcium with alizarine red S according to DAHL's method (1952). After their conversion into distinctly polygonal crystals of calcium carbonate the reaction turns out negative.

Finally, it has to be noticed that the chemical nature of the regenerating membrane itself, the matrix, is very similar to that of the organic crystals deposited upon it. Also the matrix exhibits positive reactions for proteins, i.e. collagen, and acid mucopolysaccharides. Also the character of the matrix will be discussed in more detail in another paper.

DISCUSSION

Our optical and histochemical examinations of shell regenerates in Helix pomatia show that the mineralization of the matrix is a complicated process which proceeds in two phases. In the first phase the organic crystals are formed.

They undergo growth and morphological transformations typical for the crystals of all native and synthetic organic polymers.

We can understand that BIEDERMANN at his time (1902) could not recognize the organic nature of the described primary calcifying corpuscles. He has observed many developmental stages of organic crystals, and described them as plates and spherulites. Yet, it seems that he has not had the opportunity of distinguishing between the basic types of calcifying corpuscles, and of following the sequence of morphological transformations during their growth. It also seems to us that there is no sufficient evidence for the assumption of different ways of development of the shell in embryonic, growing, and regenerating snails. In our preparations of regenerating Helix shells we have found also all forms of calcifying corpuscles described by BIEDERMANN as representative for the embryonic and the growing shell. According to BIEDERMANN the small crystalline plates show no birefringence in the shells of embryos, but are birefringent in the centre in the shells of growing animals, and strongly birefringent in the shells of regenerates. His illustrations make me think that in this case the author has compared crystals of different age, and perhaps partly confused them with the initial calcifying granules. By examining the different calcifying corpuscles in chronological sequence we found that only the finest granules are without birefringence. On growing larger, and becoming disks with one or more rings they always show birefringence almost over the whole surface. Contrary to BIEDERMANN's suggestions some decrease of the birefringence could be observed towards the centre. Also BIEDERMANN's assumption that the spherulites in the regenerating shell form a surface layer of solely emergency character, which has only a protective significance, and that the real regenerate forms under this layer appears doubtful. Our experiments showed that in regenerating shells the formation of crystals can follow different courses. Depending upon the amount of available reserves of material in the organism and the extent of the regenerating field, the growing crystals can attain the forms of large spherulites or remain quite small. Aggregates of crystals can arise both from large and from small, and from mixed forms. But by aggregation all these forms are finally converted into the polygonal crystals of calcium carbonate which constitute the base for the definitive growth of the shell. Finally, I can not agree with BIEDERMANN that only the epithelium of the mantle edge possesses the ability of producing a normal shell, and that the epithelium of any other part of the mantle can produce only irregular inner layers of the shell. The secrete produced by the mantle is liberated in the space between the mantle and the shell in the form of the so-called pallial fluid, and can be transported to all parts of the shell, or (and) to all places of damage. The contact with the epithelial cells does not seems to have any decisive significance for the formation of the shell. In this connection the work of HIRATA (1953) is worthy of notice. This author worked with isolated

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mantle-shell preparations of oysters. The shell formation was observed on pieces of glass coverslips inserted between mantle and shell. HIRATA found that all parts of the mantle were capable of shell formation in vitro, and that within about 2—3 days an organized shell structure began to appear. HIRATA also observed the birefringence of the crystals which he described as inorganic formations.

BEVELANDER and BENZER (1948) have analysed the calcification in regenerates of the shell of marine molluscs, using the glass coverslip insertion method, and by means of time-lapse photography. The microphotographs show that the authors have observed the initial granules as well as larger plates deposited in the organic membrane. According to BEVELANDER and BENZER the granules consist of calcium phosphate, are deposited in the matrix, and undergo a typical crystal growth. The crystals come to be enclosed in a thin layer of organic matrix, and assume a polyhedral shape. These crystals are composed of calcite. Comparison of the illustrations in this particularly interesting paper with our photographs of crystals creates the impression that in Helix the number of different types of crystals is larger, and that their growth transformations are more complicated than in the molluscs examined by BEVELANDER and BENZER. The organic nature of the early stages of crystals seems not to have been recognized by these authors.

In a recent work about shell formation in marine and fresh water molluscs BEVELANDER (1952) has applied labelled Ca^{45} and P^{32}. The author shows that the labelled calcium was rapidly incorporated into the calcium carbonate crystals in the newly formed shell; labelled P^{32}, on the contrary, was incorporated in the organic matrix surrounding the crystals. On the strength of these experiments BEVELANDER suggested as highly probable that the protein complex produced by the mantle may be actively involved in the formation and growth of the mineral crystals which come to be incorporated in the shell. There is an essential difference between the interpretations of the mineralization process given by BEVELANDER and BENZER, by BEVELANDER, and by us. The authors cited, as we understand their interpretations, take the organic matrix (periostracum) surrounding the crystals to be the active organic component of the calcifying system. Within this active surrounding the growing crystals behave rather passive. To us the active centers of mineralization are the crystals themselves which in their early phase of development are of organic nature, and only secondarily are transformed into crystals of calcite. To us the passive role seems to be played rather by the matrix forming the supporting and nourishing environment. Yet, for the time being all interpretations of the crystal-matrix relations in the calcification process have only the weight of working hypotheses. There certainly exists also the possibility, though not a very plausible one, that the calcification mechanism in Helix differs in some respect from that in marine molluscs.

Theoretically MANIGAULT's (1939) concept is the closest to our standpoint. The histological and histochemical methods used by him did, however, not permit him to observe the organic crystals themselves before their destruction. As his illustrations show, MANIGAULT has observed only the rests of the organic material around the $CaCO_3$ crystals.

On the submicroscopical structure of the organic crystals of the calcifying shell of Helix only theoretical suggestions can be made at present. Between the protein fibrils of the collagen lies the acid mucopolysaccharide as a cementing substance. They are bound together by salt-like linkages, as supposed by MEYER (1953), SHATTON and SCHUBERT (1954), and others. It is possible that there exist also other bonds between these substances. In organic crystals calcium can be attached to the acid mucopolysaccharide, probably to anionic groups of chondroitin sulfate, either to sulphuric acid-rest groups in the galactosamine, or to the free carboxyl groups in the glucuronic acid. This agrees with the hypothesis proposed by ARMSTRONG (1952) for the calcification process of the cartilage.

In the second phase of the mineralization the organic crystals are transformed into inorganic ones. This mechanism is still not clear. Our observations on the inhibition of carbonic anhydrase and shell calcification indicate that this enzyme is necessary for the formation of $CaCO_3$ crystals in the regenerating shell. In this respect our observations are in agreement with those by WILBUR and JODREY (1955). These authors reported that carbonic anhydrase inhibitors markedly reduce the rate of deposition of calcium in the shell of oysters. They expressed the opinion that the enzyme has this influence only when the rate of deposition of calcium is high. Molluscs which are depositing carbonate at a low rate would not need the enzyme. Our researches concerning the transformation of organic crystals into the inorganic ones in the shell of Helix are being continued. It seems to us already now that besides of the carbonic anhydrase probably some other enzymes, too, are engaged in this process.

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SUMMARY

1. The process of the mineralization of shell regenerating membrane—the organic matrix—was studied in Helix pomatia.

2. This process is performed in two successive phases. During the first phase organic crystals of the typical shape of polymer crystals are formed. In the second phase the organic crystals are transformed into inorganic crystals of calcium carbonate.

3. Small initial, not or slightly birefringent granules are the crystallization centres. The basic forms of the crystals are the round and boat-like disks, and the spherulites. Except for their minute central parts and the borders all

these forms show birefringence already at their first appearance. In the early round disks the structural elements are linearly arranged. Simultaneously with the transformation of the linear polarity into multipolarity the round disks turn into multilobular bodies resembling spherulites. The boat-like disks show negative birefringence with respect to their longer axis. The successive unequal growth of central and terminal parts of disks is connected with an increase of the birefringence and with a characteristic desorientation of the original pattern of interference colours. It results in the formation of coupled spherulite-resembling bodies. The true spherulites show a negative Brewster's cross with the index ellipsoides tangentially arranged. The patterns of interference colours of the most advanced stages of spherulites indicate an increasing structural differentiation with a typical gradient along the radii of every ring of the spherulite. Due to the inner spatial separation all forms of organic crystals finally yield compact needle clusters (or couples of clusters)—dendrite-like spherulites—to be transformed into polygonal inorganic crystals.

4. The organic crystals are basophilic. They remain metachromatic even after hydrolysis by RNase and after the extraction of lipids. They show staining reaction with pyronin Y after hydrolysis by RNase. They are PAS-positive. The destruction of the organic crystals is complette after successive action of trypsin and testicular hyaluronidase. The total of reactions proves that an acid mucopolysaccharide (possibly chondroitin sulfate) is present in organic crystals. The reactions with bromphenol blue and GRAM—WEIGERT's stain show also the presence of a protein, probably a collagen.

5. The carbonic anhydrase seems to be involved in the conversion of the organic crystals into the inorganic ones.

6. The mechanism of the process of mineralization is discussed.

ACKNOWLEDGEMENT

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PAPER CHROMATOGRAPHY AND BIOLOGICAL EFFECTS OF EXTRACTS OF THE SALIVARY GLAND OF NEPTUNEA ANTIQUA (GASTROPODA)

BY

RAGNAR FÄNGE

From the Department of Zoophysiology, University of Lund.

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In a preliminary communication (FÄNGE 1957) it was reported that the so-called salivary or pharyngeal gland of the marine snail *Neptunea antiqua* contains a toxic substance. Previously ASANO (1952) in Japan described food poisoning caused by the salivary gland of *Neptunea arthritica*. Extracts of the salivary gland of *Neptunea antiqua* produce acetylcholine-like effects on certain isolated organs; when extracts are injected into mice, these develop symptoms of a heavy stimulation of the parasympathetic system (FÄNGE 1957). Later it was found that the extracts also possess nicotine-like actions (EMMELIN and FÄNGE 1958).

MATERIAL AND METHODS

Living specimens of *Neptunea antiqua* were obtained from the Marine Zoological Station, Kristineberg, on the west coast of Sweden. The shells were broken by means of a screw-vice. The fresh glands were collected in pure acetone, the volume of which was chosen to give a final concentration of about 80 % acetone.

Preparation of extracts: After grinding in a glass homogenizer the acetone extracts were filtered and concentrated by evaporation for dryness under

vacuum. If the extracts were to be used for chromatography, the residue was dissolved into 1—3 ml of ethanol. For pharmacological experiments the residue was dissolved into 1—3 ml of distilled water.

Paper chromatography: The method of AUGUSTINSSON and GRAHN (1953) for the separation of choline esters was used. Ascending and descending chromatograms were run on Whatman no. 1 filter paper. Biological testing of the chromatograms was carried out by placing 5 mm paper strips from the chromatograms in an organ bath with a suspended *Buccinum* radula-muscle (FÄNGE and MATTISSON 1958). By this method the area of the chromatograms which contained the *Neptunea* poison was easily localized (Fig. 2). As the spray reagent, a solution of dipicrylamine was used (ACKERMANN and MAUER 1943, AUGUSTINSSON and GRAHN 1953). Exposure to iodine vapour was also used to reveal the spots.

Biological effects: Use was made of the *Buccinum* radula-muscle (FÄNGE and MATTISSON 1958) and the *Cyprina* heart (WELSH 1955). A few experiments were performed on intact animals: small fish (*Ctenolabrus, Gadus*) and laboratory mice. The extracts were injected intramusculary (fish) or intraperitoneally (mice).

Substances used: Acetylcholine, choline, neurine, acetyl-β-methyl-choline (mecholyl), urocanylcholine (murexine) and benzoylcholine. Concentrations of acetylcholine (chloride) and neurine (bromide) are expressed in the text below as g/ml suspension fluid. All values refer to the salts.

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RESULTS

THE SALIVARY GLAND

The salivary gland is a large paired organ situated at the base of the proboscis. In 12 medium-sized animals the weight of the organ varied between 0.5 and 3.4 g and on the average was about 4 % of the body weight without the shell. Probably the gland is innervated by secretory nerves, because during the dissection of large individuals there was observed on each side a fine thread leading from the cerebral ganglia to the gland.

When cuts were made in the fresh gland tissue, a formation of gas bubbles was observed in the fluid from the tissue and an intense odor reminiscent of raw fish and rhubarb was noticed. Probably the odor was due to volatile amines. A formation of gas bubbles is also seen in the sulfuric-acid-producing salivary gland of the Mediterranean species *Cassidaria* (personal observation). Fluid collected from freshly cut gland of *Neptunea* showed a pH of 7.0 as determined by indicators. Thus there does not seem to be any production of acid as in *Cassidaria, Dolium* etc.

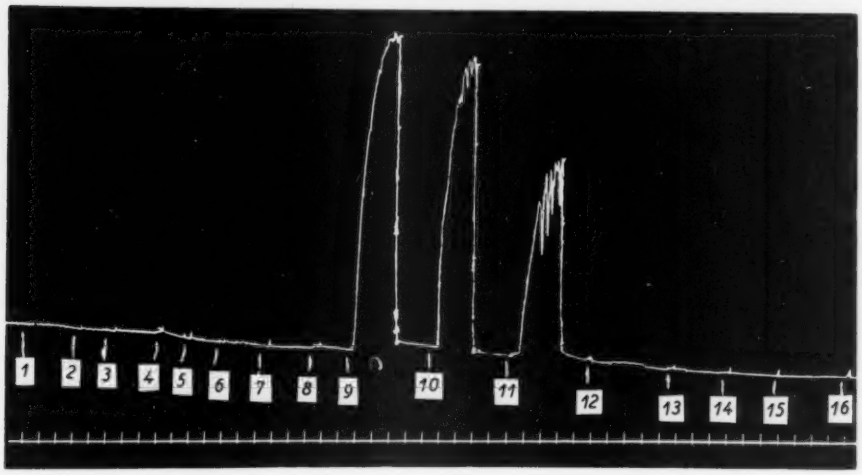

Fig. 1. Bioassay (radula-muscle) of a chromatogram of *Neptunea*-extract. 5-mm strips of the filter paper were placed in the bath. Only the strips 9, 10 and 11 at a distance of 5 cm from the point of application produced contraction. Between each single test the chymograph was stopped and the bath fluid renewed. (This chromatogram was made with an alkaline solvent and not the acid one usually used). Time indication: 60 seconds.

PAPER CHROMATOGRAPHY

On biological testing of paper strips from the chromatograms I always found a distinct area (R_f about 0.35; ascending method) containing strong contracting activity (Fig. 1). When chromatograms were sprayed with dipicrylamine solution a corresponding area (R_f 0.29—38; ascending method) developed an intensive brick-red colour. The same spot could also be revealed by exposure of the chromatograms to iodine vapours.

Because extracts of the *Neptunea* gland produce acetylcholine-like effects, I first thought that the poison might be a choline ester. When, however, a series of chromatograms were run using choline and different choline esters as reference substances, the active spot could not be identified with any of the spots produced by the reference substances.

The R_f of the active spot lay between those of acetylcholine and choline and very closely resembled that of choline (Fig. 2, A). With dipicrylamine the area containing the contracting substance stained red like the choline spot whereas the choline esters gave a yellow-brown tint. In order to obtain a definite separation between the *Neptunea* poison and choline, it was necessary to run the chromatograms for as long as 2—3 days (descending method). On these long-time chromatograms the *Neptunea* substance had clearly moved a little more rapidly than choline (Fig. 2, B).

At this stage in the investigation I abandoned the idea that the *Neptunea*

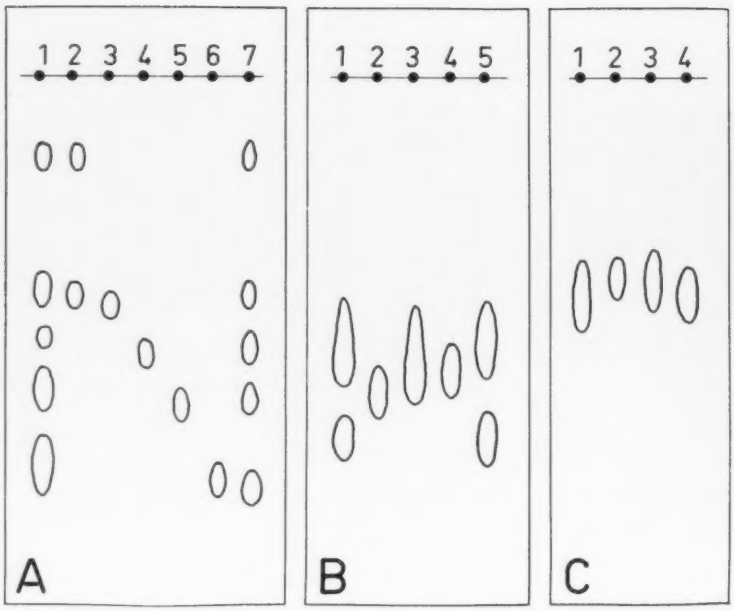

Fig. 2. Paper chromatograms of *Neptunea*-extracts and different acetylcholine-like compounds *A* (ascending, 24 hours): 2, urocanylcholine and *Neptunea*-extract; 3, choline; 4, acetylcholine; 5, mecholyl; 6, benzoylcholine; 1 and 7, mixtures of all compounds. *B* (descending, 48 hours): 1, acetylcholine and choline; 2, *Neptunea*-extract; 3, *Neptunea*-extract and choline; 4, *Neptunea*-extract; 5, acetylcholine and choline. *C* (descending, 36 hours): 1, *Neptunea*-extract and neurine; 2, *Neptunea*-extract; 3, neurine; 4, *Neptunea*-extract and neurine.

substance was a choline ester in favour of the guess that it might be a quaternary ammonium base chemically related to choline. Because preliminary experiments had shown that the substance in addition to muscarinic effects also had powerful nicotinic effects it was decided to study neurine (trimethyl-vinyl--ammonium hydroxide), which is highly toxic and possesses both muscarine and nicotine actions (HUNT 1926).

When solutions of neurine and gland extracts were chromatographed side by side or mixed, it was impossible by the chromatography method used to separate the two spots from each other (Fig. 2, C). After treatment with dipicrylamine, the neurine showed exactly the same brick-red colour as the active substance of the *Neptunea* gland.

In some of the chromatograms of gland extracts there appeared after spray with dipicrylamine solution a very faint spot with the same R_f value as acetylcholine. No attempts were made to determine whether the spot was due to acetylcholine or to any other substance.

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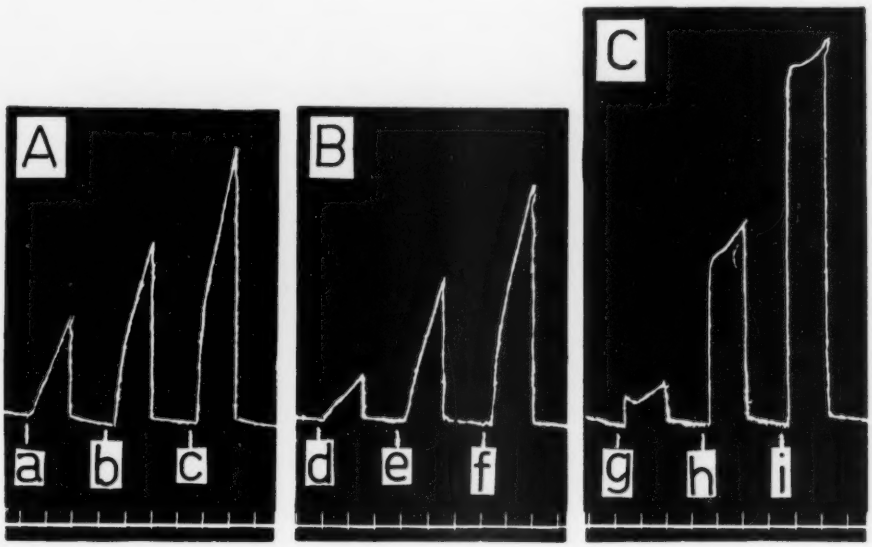

Fig. 3. Comparison between the effects on a *Buccinum* radula-muscle of solutions of neurine 0.5×10^{-3} (A), *Neptunea*-extract 10^{-2} (B) and acetylcholine 10^{-5} (C). Millilitres of the solutions added were: a, 0.10; b, 0.15; c, 0.20; d, 0.10; e, 0.15; f, 0.20; g, 0.05; h, 0.10; i, 0.15. Volume of the bath 35 ml. Temperature 18° C. Time indication: 60 seconds.

BIOLOGICAL EFFECTS

Fish. In specimens of *Ctenolabrus* or *Gadus*, which had received injections of gland extracts, the body muscles around the site of injection went into contracture and the animals swam in a peculiar stiff and curved body posture. A dose corresponding to 1/100—1/10 of a *Neptunea* gland often had a deadly effect. If the injected fish did not die within the first 15 minutes, recovery took place within 3—4 hours.

Mice. A dose corresponding to about 50 mg of fresh gland was fatal. Somewhat smaller doses produced heavy secretion of saliva and tears, but the animals recovered. The secretory effects were abolished by atropine. — The fatal dose of neurine (base) was found to be ca. 0.045 milligrams per gram mouse. This means that if the toxic effect of the *Neptunea* gland is due to neurine, the tissue contains approximately 3 % neurine.

Buccinum radula muscle. In fig 3 the actions from different doses of *Neptunea* gland extract, neurine and acetylcholine are compared. The shape of the contraction curves obtained with *Neptunea* extract and neurine seemed to be of the same type as that obtained with acetylcholine after eserinization (Fänge and Mattisson 1958). It is seen from the results illustrated in Fig. 3

that the gland extract in its actions resembled a 3—5 % solution of neurine bromide (which corresponds to about 2 % of the base).

The contracting effect of the extracts was not visibly potentiated if the radula-muscle had been pretreated with eserine. In this respect, as also in the shape of the contraction curves, the *Neptunea* substance differs from acetylcholine.

Cyprina heart. Isolated *Cyprina* hearts were stopped in diastole by a dilution of 10^{-3} of the extracts.

STABILITY OF THE SUBSTANCE

Boiling for a few minutes at pH 9.0, which completely decomposes acetylcholine, did not destroy the activity of the extracts. Addition of a drop of fresh human blood to the extracts had no inactivating effect. Thus the substance is not inactivated by cholinesterase.

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DISCUSSION

There are several examples of gastropods which use poisonous secretions to paralyze or kill their prey. *Poiretia* attacks lumbricids (WÄCHTLER 1927), *Dolium* and *Cassidaria* echinoderms (SIMROTH 1907), *Conus sp.* polychaetes (ALPERS 1932) or fish (KOHN 1956) etc. The presence in the salivary gland of *Neptunea* of a strong poison makes it possible that it obtains its animal diet by the poison method. In addition *Neptunea* as other buccinids is certainly a carcass feeder also. In an aquarium I have occasionally seen that it ingests dead crabs. In the natural surroundings of *Neptunea* among other animals the following occur: *Turitella, Pecten opercularis, Ophiothrix, Echinus esculentus, Verruca strömi, Telepus cincinnatus,* serpulids. During the present investigation I found that newly caught specimens often had their intestine filled with a red pigmented mass, apparently emanating from some red coloured animal ingested. Undoubtedly a closer examination of the intestinal content would give information concerning the food habits of *Neptunea.*

The salivary glands of different gastropods seem to produce different products. In *Buccinum* they contain a substance resembling 5-hydroxytryptamine (WELSH 1956). In *Busycon* a trypsin-like enzyme has been found (MENDEL and BRADLEY 1905). *Dolium, Cassidaria* and a few other Mediterranean species are well known for their peculiar production of a sulfuric acid-containing saliva. According to my own observations acetone extracts of the acid gland of *Cassidaria* has 5-hydroxytryptamine-like effects. The chemical nature of the highly toxic substances produced by *Conus* species (IREDALE 1935) seems to be quite unknown. Urocanylcholine, which occurs in *Murex* (ERSPAMER 1952) and

Urosalpinx and *Thais lapillus* (WHITTAKER and MICHAELSON 1954) seems to be produced by the hypobranchial and not the salivary gland.

If the toxicity of the *Neptunea* salivary gland is due to neurine, the gland must contain several per cent of this substance, which seems astonishingly high. The effects of the extracts could also be explained by the presence of a substance closely related to neurine but more active.

SUMMARY

Extracts of the salivary glands of *Neptunea antiqua* contain a toxic substance. The results from paper chromatography and from studies of the biological effects of the extracts make it likely that the active substance is a quaternary ammonium base, possibly neurine or a compound related to neurine.

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Acknowledgements.

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COMPARISON BETWEEN BIOLOGICAL EFFECTS OF NEURINE AND A SALIVARY GLAND EXTRACT OF NEPTUNEA ANTIQUA

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Previous experiments have shown that salivary gland extracts from *Neptunea antiqua* contain an agent with muscarine-like actions; in paper chromatograms the active substance behaved like neurine (FÄNGE 1957). In the present investigation a comparison was made between gland extracts and neurine (bromide) on different biological test objects.

METHODS

An ethanol extract of the salivary glands was prepared as described earlier (FÄNGE 1957). One ml of extract corresponded to about 2 g of gland tissue. The following test objects were used.

1. The frog rectus. The muscle was suspended in a 2 ml bath containing oxygenated frog Ringer solution.

2. The frog heart, isolated according to Straub. The cannula contained 2 ml frog Ringer solution.

3. The cat's blood pressure. The cats were anaesthetized with chloralose (about 80 mg/kg intravenously after preliminary ether). The blood pressure was recorded in a carotid artery using a mercury manometer. Drugs were injected through a cannula in a femoral vein. In order to study if the drugs released catechol amines from the suprarenal medulla injections were also made into the coeliac artery in retrograde direction after ligation of the superior mesenteric artery.

4. Secretion from the submaxillary gland of the cat. A cannula was intro-

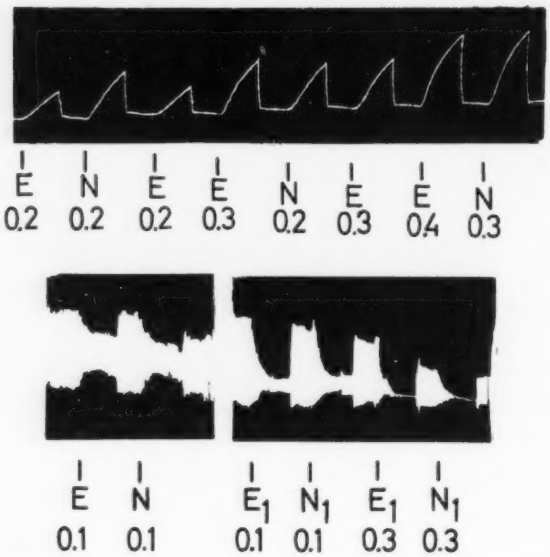

Fig. 1. Upper record: frog rectus in 2 ml bath. E = extract of salivary gland, diluted 1 : 100. N = solution of neurine bromide 1 : 1000. The volume of each dose, in ml, is given Lower record: isolated frog heart, 2 ml bath. E = extract 1 : 100, E_1 = extract 1 : 10. N = neurine bromide 1 : 1000, N_1 = neurine bromide 1 : 100.

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duced from the mouth into the submaxillary duct. Drops of saliva falling from the tip of the cannula were recorded on the smoked drum using an electrical signal operated by an assistant.

5. The denervated tongue of the cat. The muscles of the tongue had been sensitized to nicotine-like substances by section of the hypoglossal nerve a week in advance. Contractions of the tongue were recorded by a lever connected to the tip of the tongue via a thread, a pulley and a clip.

5. The respiration of the cat. The tracheal cannnula was connected to a tambour.

RESULTS

Fig. 1 demonstrates that the extract contracts the rectus muscle and inhibits the frog's heart. Neurine has the same effects. Both assays indicate that the activity of the original extract corresponded roughly to a 10 p.c. solution of neurine bromide.

In Fig. 2 the blood pressure, the salivary secretion and the contractions of the tongue were recorded. It can be seen that both the extract and neurine caused a small lowering followed by a more pronounced increase of the blood

pressure. With bigger doses the pressor responses dominated. Both agents evoked a flow of saliva and a contraction of the tongue. A quantitative comparison shows that the extract in this instance also corresponded to a 10 p.c. solution of neurine bromide in all the three test objects.

The initial fall in blood pressure was found to disappear after injection of atropine. The rise was diminished by big doses of nicotine. The salivary responses, on the other hand, were not affected by previous big doses of nicotine; they were abolished by atropine. Flow of saliva was obtained after injection of extract or neurine even after section of the chorda tympani. Contractions of the denervated tongue were smaller after previous injections of big doses of nicotine.

TABLE I

Volume injected (ml)	Drops of saliva in response to	
	Extract 1 : 10	Neurine 1 : 100 (bromide)
0.05	0	0
0.1	1	1
0.2	8½	8⅔
0.3	16	15⅔

The Table 1 shows an assay of the activity of the extract on the submaxillary gland. Four different doses of extract and of neurine were given. It is obvious that on increasing the doses the effects of both agents increased in parallel.

The effects of the two agents injected close arterially into the suprarenals are shown in Fig. 3. For comparison the effects of intravenous injections are shown as well. It can be seen that a dose of extract which caused a small effect on the blood pressure when given intravenously gave a pronounced effect on close arterial injection. This strongly suggests that the effect in the latter case was due to liberation of catechol amines. Neurine had a similar effect.

In the experiment of Fig. 4 the effects on the respiration (and the secretion of saliva) was recorded. The two agents were found to cause a pronounced but short-lasting increase in the respiratory movements. With big doses this augmentation was followed by a reduced rate of respiration, probably secondary to the raised blood pressure. The increase in respiration, on the other hand, was obviously not due to a fall in blood pressure, since it persisted after an injection of atropine, which abolished the depressor action of the two agents. The activity of the extract on the respiration (and on the salivary secretion) still corresponded to a 10 p.c. solution of neurine bromide.

In one experiment the extract was compared quantitatively with choline. Doses of choline, which had about the same effect as a certain dose of the

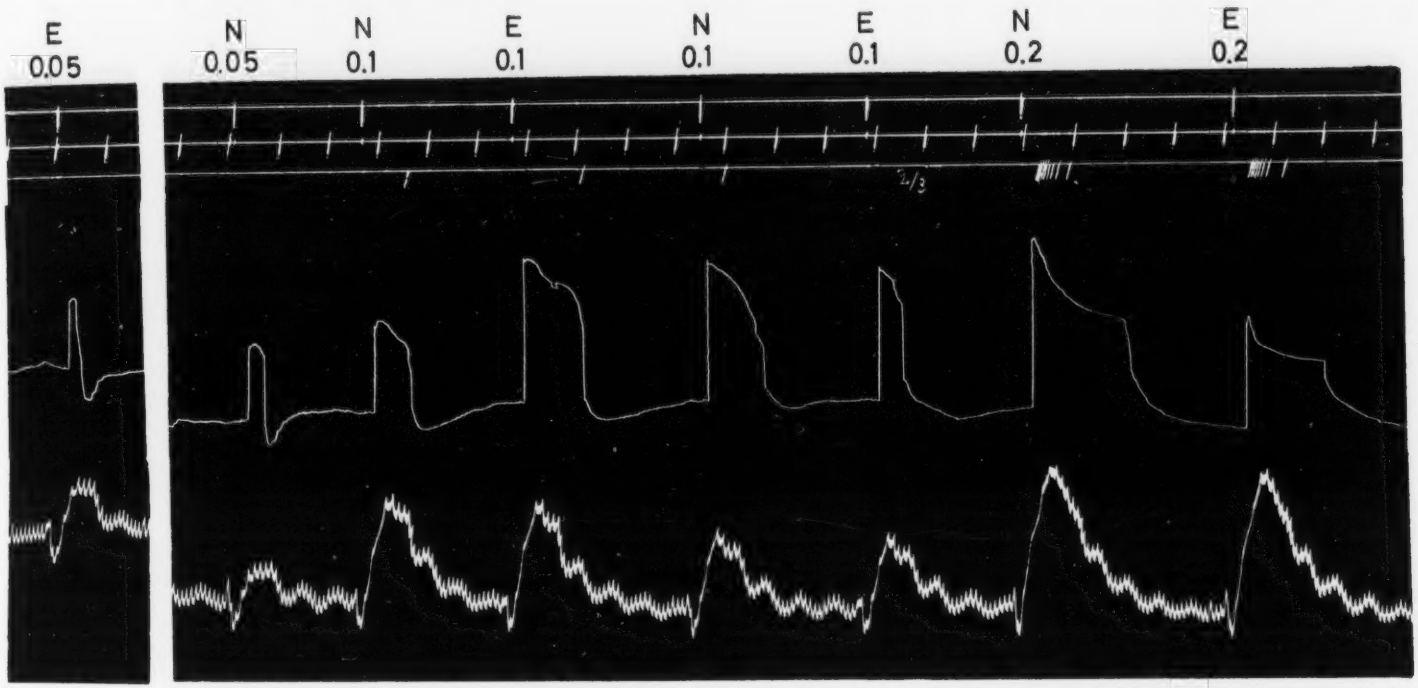

Fig. 2. Cat. Records from above: Signal for intravenous injections of extract 1 : 10 (E) or neurine bromide 1 : 100 (N); time in minutes; drops of saliva; contractions of the denervated tongue; blood pressure.

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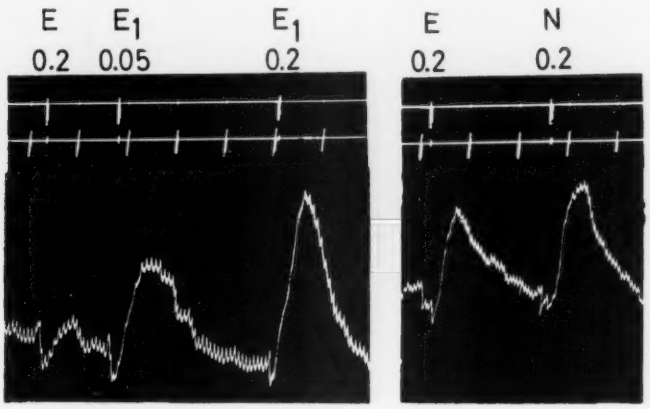

Fig. 3. Cat. From above: signal for injections of extract (1 : 100 = E, 1 : 10 = E1) or neurine bromide (1 : 1000 = N); time in minutes; blood pressure. The first three injections were given intravenously, the subsequent ones into the coeliac artery.

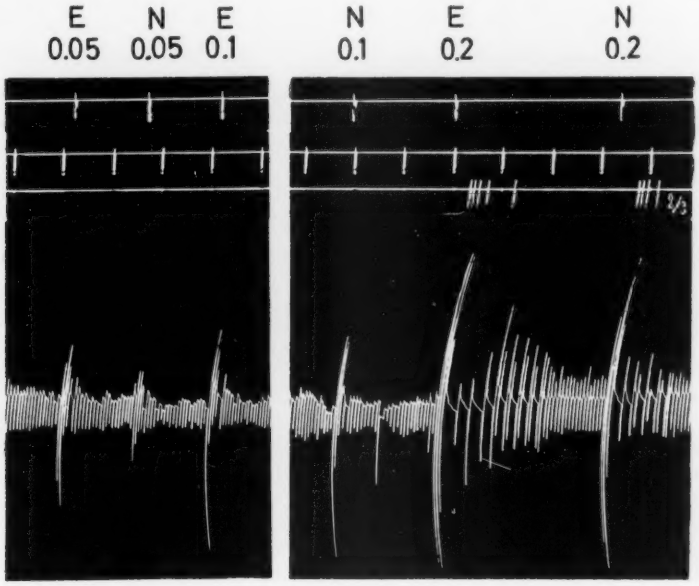

Fig. 4. Cat. From above: signal for intravenous injections of extract 1 : 10 (E) or neurine bromide 1 : 100 (N); time in minutes; salivary secretion; respiration.

extract on the submaxillary gland or the denervated tongue, were found to have a pure depressor action, whereas the dose of extract caused a big rise in blood pressure.

DISCUSSION AND SUMMARY

The present experiments show that the extract of the salivary glands of *Neptunea antiqua* possesses both muscarinic and nicotinic actions. The following muscarine-like effects have been observed: inhibition of the frog's heart; secretion of saliva in the cat, which is not abolished by section of the chorda or previous injection of big doses of nicotine but by atropine; fall in blood pressure abolished by atropine. The nicotine-like effects are: contraction of the frog rectus; contraction of the tongue muscles, sensitized by previous denervation; this latter effect is diminished by injection of big doses of nicotine; rise in blood pressure, reduced by nicotine; liberation of catechol amines; stimulation of the respiration.

Neurine has just the same properties. When a quantitative comparison was made between extract and neurine the extract was in all the test objects and in all the doses tried found to correspond to a 10 p.c. solution of neurine bromide. This strongly suggests that the active agent of the extracts is identical with neurine or a closely related compound. If so the gland should contain as much as about 25 mg of neurine per gram of tissue.

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STUDIES ON THE PHYSIOLOGY OF THE RADULA-MUSCLE OF BUCCINUM UNDATUM

BY

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INTRODUCTION

The radula-muscle in *Buccinum* is a group of muscle bundles, which are connected with the tooth apparatus (radula), and which are easily distinguished from other muscles by their red colour. RAY LANKESTER (1873) showed that the red pigment of certain molluscan muscles is haemoglobin. BALL & MEYERHOF (1940) investigated spectroscopically ironporphyrin compounds in the radula muscle of a species related to *Buccinum*, the American *Busycon canaliculatum*. These authors found that besides haemoglobin the radula-muscle contains a complete cytochrome system comprising cytochrome oxidase and the cytochromes a, b and c.

Many physiological and pharmacological studies have been made on molluscan muscles, but most of these works have been made on white muscles (anterior byssus retractor muscle of *Mytilus*, adductor muscle of *Pecten*). In a short communication HILL (1956) reported that the radula-muscle of *Busycon* starts rhythmical contractions when stimulated by acetylcholine and 5-hydroxytryptamine given simultaneously. The same observation was made by FÄNGE & MATTISSON (1958) on *Buccinum*.

In the present investigation the cellular respiration and the behaviour of the radula-muscle, when stimulated with certain physiologically active compounds, have been studied. In addition a brief study was made of the microscopic structure.

MATERIAL AND METHODS

Living *Buccinum undatum* were collected at the Marine Zoological Station Kristineberg. During the preparation of the radula-muscle the oesophagus was taken away. For a comparative purpose other kinds of muscle tissue were used in certain experiments; white molluscan muscle (*Buccinum* foot) and red mammalian muscle (pig heart).

Histological: After fixation in Bouin's fluid, paraffin embedding and sectioning (6—10 μ) the preparations were stained with azan, Ehrlich ironhaematoxylin or Giemsa.

Preparation of cytochrome c: The preparation was done according to the chromatographic method described by Boeri & Tosi (1954) with the modification, that the more finely granulated Amberlite XE-64 was used instead of Amberlite IRC-50. The cytochrome concentration of the eluate was determined spectrophotometrically.

Cytochrome oxidase activity: The activity of cytochrome oxidase was examined on the supernatant after homogenization in a 1/15 M phosphate buffer of p_H 7,2 and slight centrifugation. The determinations were done spectrophotometrically (Cooperstein & Lazarow 1951) using a cytochrome c prepared from horse heart (Keilin & Hartree 1945). The activity was calculated according to Webster's formula.

Manometric experiments: In the Warburg apparatus (B. Braun, model V) besides usual vessels holding 15—20 ml also micro-vessels with 3—5 were used. Temperature was 25° C. The tissues were finely cut by a pair of scissors. With some modifications for small vessels, the directions given by Umbreit, Burrow & Stauffer (1949) were followed. The enzyme inhibitors used were: potassium cyanide, sodium azide and carbon monoxide. The reversibility of the inhibition was shown by adding methylene blue. The effects of sodium succinate and p-phenylenediamine were studied.

Isolated muscle preparations: The isolated radula-muscle was suspended in a 30 ml bath of sea water aerated with small bubbles. The movements were recorded isometrically on a slowly moving drum (4 mm/min.). The temperature was 18° C, except when the influence from varying temperatures was studied. Doses of drugs are expressed in the text below as g/ml suspension fluid. All values refer to the salts. Substances used were: acetylcholine chloride, choline bromide, eserine salicylate, 5-hydroxytryptamine creatinine sulphate, tryptamine chloride, LSD-25.

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RESULTS

THE STRUCTURE OF THE RADULA-MUSCLE

The gross anatomy has been studied by GEDDES (1879), OSTWALD (1893) and DAKIN (1912) on *Buccinum* and by HERRICK (1906) on *Busycon*. A short histological description is given by DAKIN (1912).

Our own results agree in the main with those of DAKIN concerning the microscopical structure. The muscle may be called a smooth muscle so far as there is no cross-striation of the individual fibres, but the structure is very unlike that of smooth muscle in vertebrates, and in many respects it reminds us of striated muscle. It consists of spindle-shaped uninuclear fibres more than 100 μ long and with a diameter of 10—20 μ. The fibres are tightly packed parallel to each other forming the different bundles or muscles, which compose the radula--muscle. Presumably the muscle fibres stretch themselves from the point of origin to the insertion point along the whole length of the bundles. In cross sections it was seen that the larger bundles contained one or more central canals or blood lacunes. In the azan stained sections we noticed that the bundles of muscle fibres as a rule were not surrounded by connective tissue membranes. The muscle fibres are stained red by azan. The central endoplasm is full of mitochondria (Fig. 1 B) whereas the dense ectoplasm contains the myofibrils. These are parallel to each other and run spirally along the fibres. The myofibrils were distinctly seen in sections stained with Giemsa, using an oil immersion objective (Fig. 1 A). In preparations stained with azan or iron haematoxylin they were less apparent. The "double oblique striation" caused by the parallel arrangement of the myofibrils has been observed in the smooth muscles of many different types of invertebrates (BALLOWITZ 1892, HANSSON 1957).

Nerves were seen widely distributed among the muscle bundles. Thus it seems reasonable that the muscle bundles are richly innervated. It would be interesting to study the motor innervation of the radula-muscle by special nerve stains.

The complex structure of the radula-muscle, composed, as it is by a lot of smaller individual muscle bundles, certainly allows a richly diversified scheme of movements of the radula and the proboscis of the whelk. By preparing radula-muscles it was seen that some muscle bundles made fibrillary twitches while others were motionless.

CELLULAR RESPIRATION

Cytochrome c.

Radula-muscles with a wet weight of 6.9 g (from about 50 animals) were used for the preparation of cytochrome c. The orange-red eluate showed marked

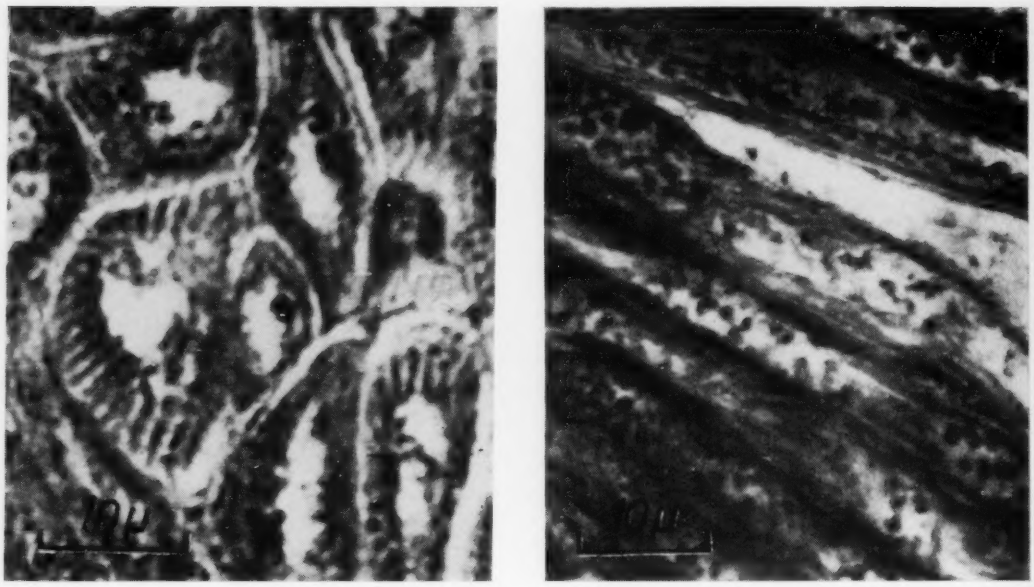

Fig. 1. *Buccinum* radula-muscle. *A*. Transverse section showing cross-sectioned ribbon-like myofibrils in the ectoplasm of muscle fibres. *B*. Longitudinal section. The endoplasm is filled with mitochondria. The myofibrils of the dense ectoplasm are indistinctly seen. Giemsa stain. Oil immersion.

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absorption peaks at 550 and 520 mμ. A similar preparation performed on 140 g *Buccinum* foot muscle also, though very faintly, showed the absorption peaks characteristic for cytochrome c. As seen in Table 1 the content of cytochrome c was about 100 times larger in the radula-muscle than in the foot muscle. When the cytochrome c content of the radula-muscle was compared with that of the pig heart, the pig heart was found to contain about 10 times more cytochrome c (Table 1).

Table 1.

Material	Cytochrome-oxidase activity *	Cytochrome c mg/kg wet weight	q_{O_2} **
Buccinum radula-muscle	0.66 (3)	20	620
Buccinum foot	0.05 (2)	0.1	120
pig heart	5.60 (3)	238	480

* log [ferrocytochrome c] per minute for a 1 : 100 tissue dilution at 25° C.
** μl of oxygen consumed per g of wet weight of tissue per hour at 25° C.
The figures within brackets are the number of experiments averaged.

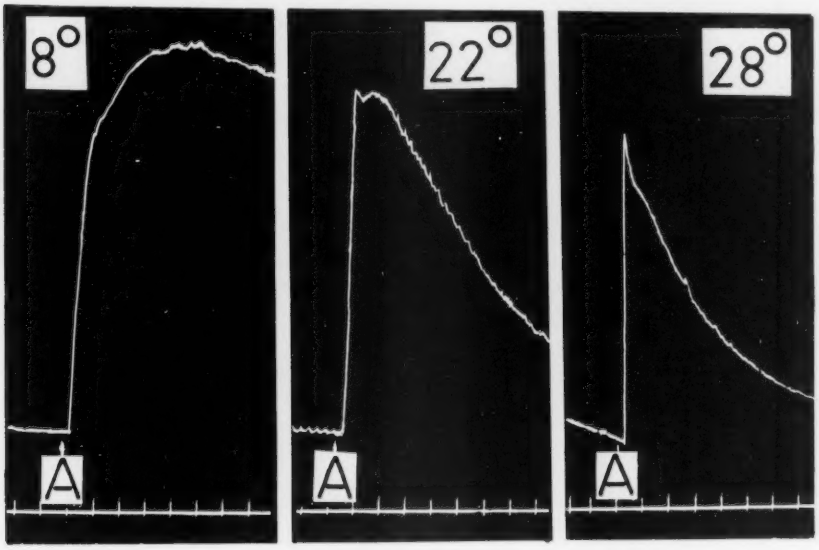

Fig. 2. The shape of the acetylcholine contraction curve at different temperatures. A = acetylcholine. At 8° C the final acetylcholine concentration in the bath was 10^{-5} g/ml; at 22° C and 28° C the conc. was 2×10^{-5} g/ml. Time indication 60 sec.

Cytochrome system and repiratory intensity.

Not only the content of cytochrome c but also the cytochrome oxidase activity of the radula-muscle is about ten times less than corresponding value of pig heart. In Table 1, besides these figures, there are also represented values for the respiratory intensity, expressed as μl oxygen consumption per g wet weight per hour. It is interesting to see that in spite of its lower cytochrome c content and lower cytochrome oxidase activity the radula-muscle showed a respiration of the same rate or even higher than that of the pig heart. It was thought that the explanation to this discrepancy might be found in a special organization of the cellular oxidation in *Buccinum*. In order to investigate the terminal oxidation in the *Buccinum* radula-muscle the effects of different inhibitors and substrates were tested.

Inhibitors.

The experiments with cyanide revealed that the respiration was highly resistant to cyanide (Table 2). The azide inhibition also was small at p_H 7. The effect of azide was only tested at this p_H and not at p_H 5 because it was found that mere lowering of the p_H caused a marked decrease in the respiration. No inhibition was obtained with carbon monoxide either in light or in darkness (95 % CO + 5 % O_2 and control vessels with 95 % N_2 + 5 % O_2).

Fig. 3. The combined effect of acetylcholine (A) and tryptamine (T). The acetylcholine concentration in all three was 4×10^{-8} g/ml. The tryptamine concentrations are given in the Figure. The preparation showed rhytmical activity after acetylcholine alone had been added, but the rhythmicity was increased by small doses of tryptamine. Time indication 60 sec.

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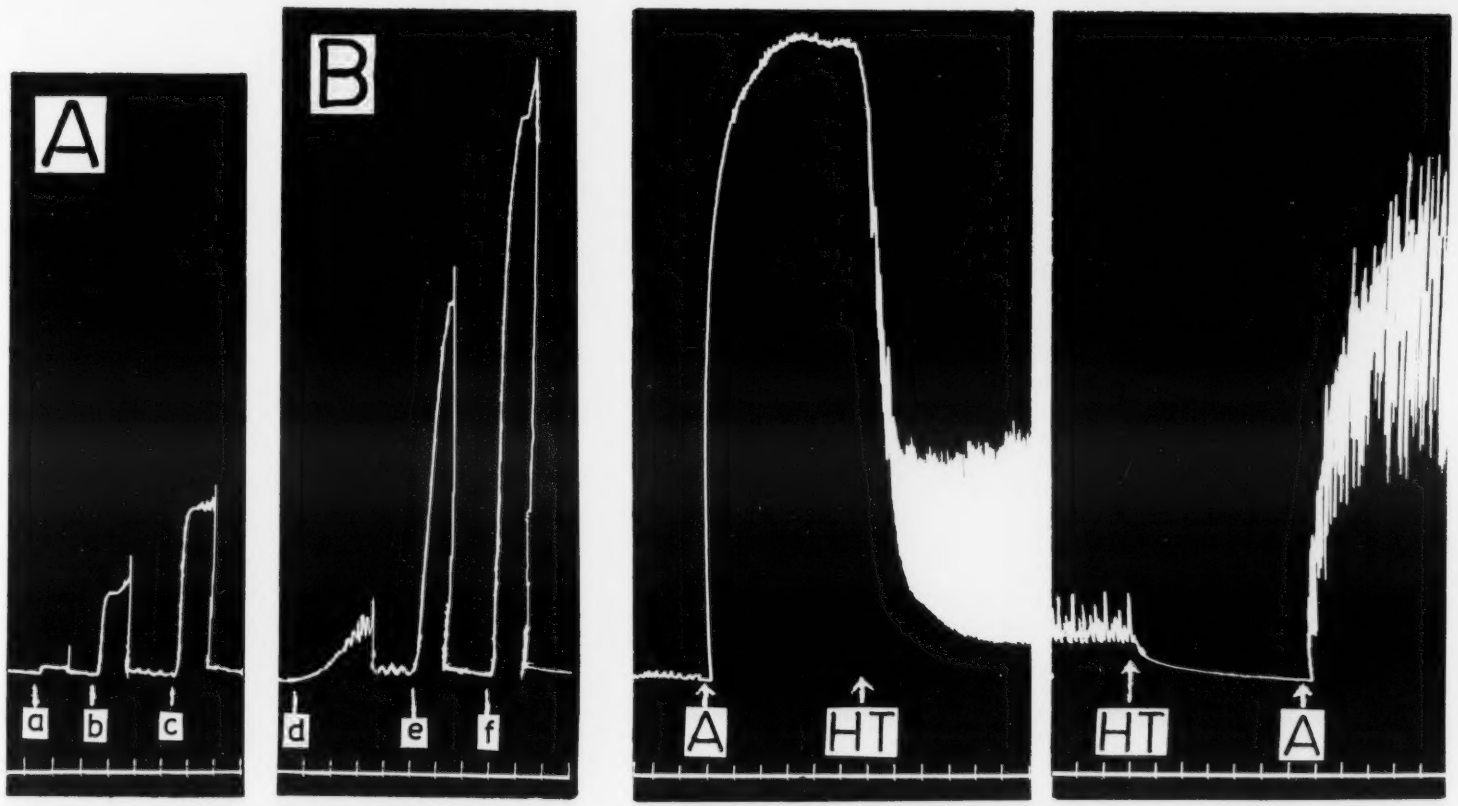

Fig. 4. *A and B:* The response to different doses of acetylcholine before (A) and after (B) treatment with eserine (10^{-5} g/ml 15 min). The following doses were given: a, 2×10^{-8}; b, 3×10^{-8}; c, 4×10^{-8}; d, 10^{-8}; e, 2×10^{-8}; 3×10^{-8}. *The two right hand curves:* Rhythmical activity produced by the combined action of acetylcholine and 5-hydroxytryptamine. Left: 5-HT was given after acetylcholine. An immediate relaxation was followed by strong rhythmical movements. Right: the same muscle preparation after washing. Small rhythmical movements persisted but these were abolished by 5-HT. When acetylcholine was added, a contraction occurred and the rhythmicity began again. Final concentration of acetylcholine 5×10^{-8}; of 5-HT 3×10^{-7}. Time indication 60 sec.

Table 2.

Effect of inhibitors and substrates on the respiration of the radula-muscle.

Sodium-succinate 4×10^{-2} M	Cyto-chrome c 10^{-5} M	KCN 10^{-2} M	KCN 10^{-3} M	NaN_3 10^{-2} M	Methylene-blue 5×10^{-3} M
				−32 (4) →	+18 (4)
			−15 (2)	——→	+20 (2)
		−42 (6)	——→	——→	+98 (6)
+37 (2)	→ +20 (2)	——→	−57 (2)		
+32 (1)	——→	——→	−48 (1)		

The concentration values in the head of the Table represent final concentrations. The figures tabulated are percentage changes of respiration, calculated on the figures of the preceeding 30 minute periods. The substances were added in the order from left to right. The figures within brackets are the number of experiments averaged.

Substrates.

When succinate or p-phenylenediamine was added to the cut tissue, the uptake of oxygen distinctly increased. A further respiratory increase above that caused by succinate or p-phenylenediamine appeared when in addition cytochrome c was supplied in spite of its low permeability. Cyanide caused a more pronounced inhibition of this increased respiration. An analogous result was obtained with azide.

THE EFFECT OF DRUGS

Initial relaxation period.

Newly suspended radula-muscles were usually in a state of tonic contraction but slowly relaxed during the next 1/2—2 hours. Until this initial relaxation had occurred it was impossible to obtain good responses to drugs added.

Acetylcholine.

Acetylcholine in concentrations from 10^{-8}—10^{-7} caused contraction. The shape of the contraction curve and the sensitivity varied according to the temperature (Fig. 2 A). Within the concentration interval of about 10^{-8}—10^{-6} the responses were proportional to the dose of acetylcholine. Treatment with eserine (10^{-5} g/ml 15—30 min.) increased the sensitivity to acetylcholine a few times and the shape of the contraction curve changed (Fig. 3). Both eserinized and non-eserinized preparations relaxed completely within 1—3 minutes when the suspension fluid was renewed. On account of the rapid and reversible reactions of the radula-muscle to acetylcholine, it was found to be of value for bioassays of acetylcholine and similar substances (FÄNGE 1958).

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Choline caused similar responses as acetylcholine but was about 1000 times less active.

5-Hydroxytryptamine.

This drug in concentrations from 10^{-9}—10^{-8} produced relaxation and rhythmical movements of radula-muscles contracted by acetylcholine or choline. The same effects were obtained with tryptamine (Fig. 2 B) and LSD-25.

In certain concentrations the simultaneous presence of acetylcholine and 5-hydroxytryptamine in the bath gave rise to rhythmical contractions, which were rapid, strong and regular and highly resembling cardiac pulsations (Fig. 4). The frequency of these contractions was 20—30 per minute and they continued 1—2 hours or more until the muscle was exhausted. After 5-hydroxytryptamine or tryptamine an increased tendency to rhythmical movements persisted in spite of several washings.

DISCUSSION

The radula-muscle belongs to that type of smooth muscle which is found in many different groups of invertebrates, the typical feature of which is a "double oblique striation". The fibres of the radula-muscle are characterized by an abundance of mitochondria and by a red colour due to haemoglobin. In resemblance to striated muscle its fibres stretch from the origin to the insertion point.

It is probably of great importance for the living *Buccinum,* that the disintegration of the food by means of the radula is a rapid process, because the whelk takes its food in competition with other carnivorous animals. In accordance with this the muscle-apparatus moving the radula has the power of exerting a very intensive activity during a limited time. The richness in respiratory enzymes localized in the mitochondria is in good agreement with such a functional ability.

In *in vitro* experiments the radula-muscle shows a remarkably high oxygen consumption. Thus in the living animal the radula muscle needs a large oxygen supply. The haemoglobin of the radula muscle can presumably act for a short time as an oxygen store. It would be interesting to investigate if and how the functional capability of the isolated radula-muscle is influenced by variations in the oxygen concentration of the suspension fluid or by putting the haemoglobin out of action by means of carbon monoxide.

When using inhibitors the interpretation of the results has many difficulties. Yet such experiments can be of great value for obtaining a knowledge of the respiratory enzyme systems. In the radula-muscle the presence of a cytochrome

system, as observed by BALL & MEYERHOF, has been confirmed. The capacity of this system is, however, low. Contrary to pig heart muscle and even to the white muscle of crustaceans (MATTISSON, unpublished results) the respiration of the radula-muscle is relatively insensitive to inhibitors of cytochrome oxidase. These facts might indicate, that the radula-muscle is provided with other terminal oxidation systems besides the cytochrome system.

Whereas 5-hydroxytryptamine in vertebrates has a contracting effect upon smooth muscles, it exerts a relaxing effect on muscles of molluscs (TWAROG 1954, HOYLE & LOWY 1956, HILL 1956; the present investigation). Perhaps such an effect is also found in other groups of invertebrates. According to our unpublished results the retractor muscles of *Phascolosoma* are relaxed by 5-hydroxytryptamine. HOYLE & LOWY (1956) found that the anterior byssus retractor muscle of *Mytilus* after treatment with 5-hydroxytryptamine, although completely relaxed, nevertheless is able to make powerful contractions when stimulated electrically. That the radula-muscle, after treatment with 5-hydroxytryptamine, is able to contract, is seen from the powerful rhythmical activity produced by acetylcholine and 5-hydroxytryptamine given simultaneously. 5 Hydroxytryptamine in low doses has very marked effect on molluscan heart (ERSPAMER & GHIRETTI 1954, FÄNGE 1955). In this case also the effect seems to involve a relaxation, because the often enormous increase in amplitudes of the heart beat is for a large part caused by a more complete diastolic relaxation.

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ACKNOWLEDGEMENT

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SUMMARY

1) The radula-muscle of *Buccinum* recalls in certain respects striated muscle. It is composed of several smaller muscle bundles, each consisting of tightly packed, very long fibres. Probably the fibres stretch from the origin to the insertion point of each muscle bundle. The ectoplasm of the fibres contains spirally arranged myofibrills and the endoplasm contains the nucleus and

numerous mitochondria. The red colour of the fibres is mainly due to a haemoglobin.

2) The content of cytochrome c is about 100 times larger than in white muscles (foot muscle) from the same species. The capacity of the cytochrome system estimated as the content of cytochrome c and the cytochrome oxidase activity is considerably lower than that of pig heart.

3) In spite of the low capacity of the cytochrome system the respiration of the radula-muscle measured in the Warburg apparatus is even higher than that of pig heart.

4) The respiration shows a remarkable resistance to cyanide, azide and carbon monoxide.

5) The radula-muscle is contracted by acetylcholine and relaxed by 5-hydroxytryptamine. Strong rhythmical contractions of long duration appear when acetylcholine and 5-hydroxytryptamine are added simultaneously. Tryptamine and LSD-25 have similar effects as 5-hydroxytryptamine.

6) In the living animal the normal way of working probably is rhythmical contractions. The high respiratory rate and the presence of haemoglobin in the muscle fibres presumably are correlated with the pronounced capacity for continuous strong rhythmical activity.

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ON THE CONE MOSAIC IN THE RETINA OF *PARUS MAJOR*

BY

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INTRODUCTION

Since the middle of the 19th century it has been known from the descriptions of HANNOVER (1843) that in many animals the double and single cones of the retina may form a definite pattern. The cone mosaic has since been examined in a number of animals by different methods. Most of these investigations were performed on fishes, and the results were summed up by LYALL in 1957. From this it may be gathered that the most common pattern among teleosts is that of a square, the double cones forming the sides, and a single cone being situated in the middle of the square; sometimes one single cone is also found in each corner (pattern *D* or *G* of EIGENMANN & SHAFER, 1900, cf. Fig. 1).

Also in reptilians, visual cell patterns are found, mainly consisting of alternating rows of single and double elements (DETWILER 1923, UNDERWOOD 1951).

In the human retina, as in that of all placentals, there are no double cones, but the single cone pattern has been described by some authors (e.g. SCHULTZE 1866, HEINE 1901, POLYAK 1941), who found an organization of the cones in regular rows. The same single cone pattern has been found in the rhesus macaque (POLYAK 1957).

However, the visual cell patterns of the bird retina are but little known. Here investigations have only been performed on the fresh, extended retina, chiefly for the purpose of mapping the distribution of the oil droplets and of examining the numerical relations between the oil droplets of different colours. Thus HANNOVER (1843) described the distribution of the oil droplets of different colours in *Gallus*, examining the fresh retina from which the pigment epithelium had been removed. SCHULTZE (1866), using the same method, examined the presence of different kinds of visual cells in *Gallus, Columba, Falco*

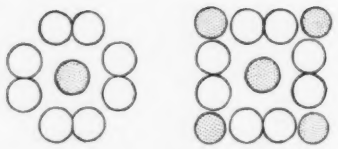

Fig. 1.

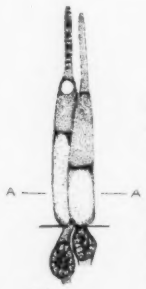

Fig. 2.

Fig. 1. The two most common cone patterns (D and G) in the teleost retina as described by Eigenmann & Shafer (1900).

Fig. 2. Avian double cone. (The more peripheral version, which is plumper and more easily studied than those in the centre. After Walls, 1942.)

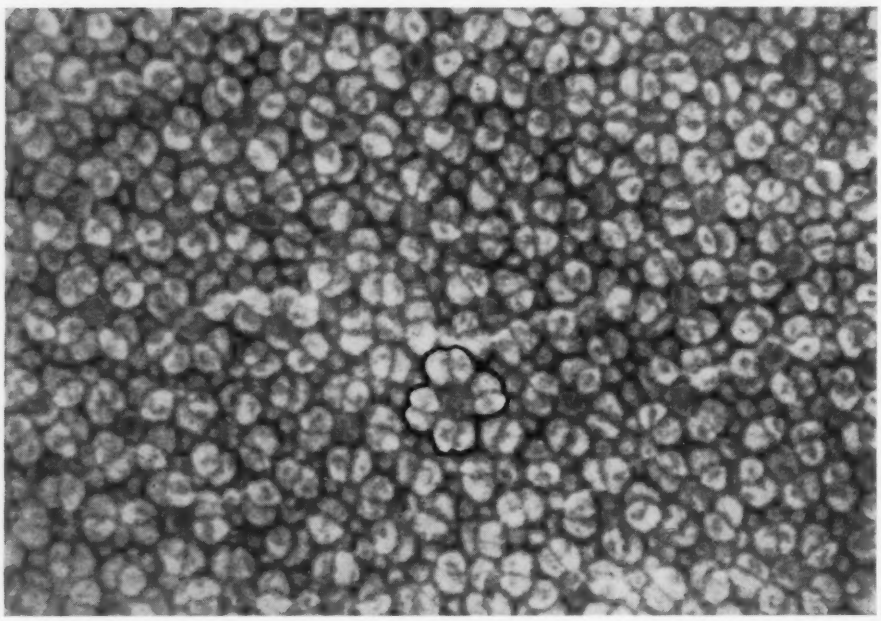

Fig. 3. Tangential section of the retina of *Parus major* on the level A—A of Fig. 2, showing the cone pattern. The outlines of one group are traced. General fundus. Regaud's haematoxylin, 6 μ. 2,000 ×.

and *Strix,* without finding any definite visual cell pattern. Later similar investigations were performed by Waelchli (1883), Hahn (1916) and others. However, investigations of the cone pattern of the bird retina based on tangential sections through the retina—the method used in the cited works on the fish retina—do not exist. Fritsch (1911), it is true, used this method in his investigations of the fovea of some birds, but he did not use the slides obtained to describe the cone pattern. In order to bridge the gap in our knowledge of the

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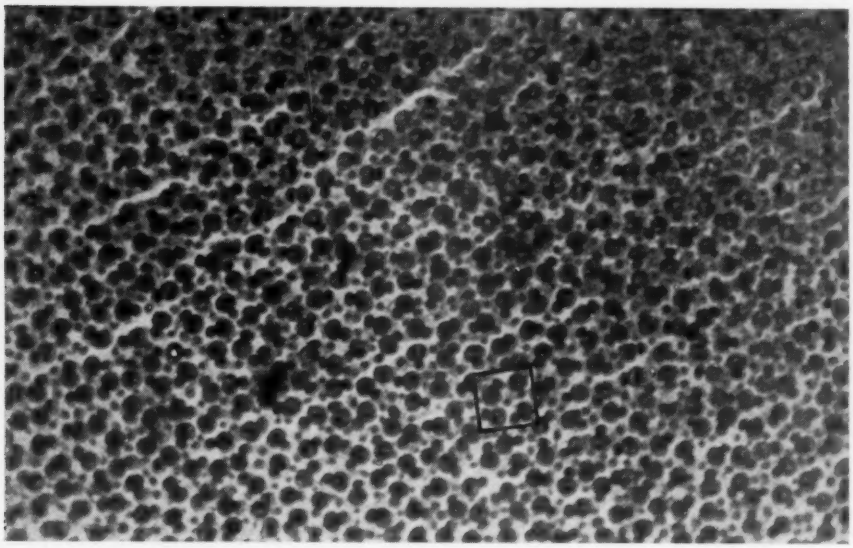

Fig. 4. Tangential section of the retina of *Parus major* through the cone nuclei just inside the external limiting membrane, which is seen in the upper, right corner. The nuclei of one group are enclosed in a square. Regaud's haematoxylin, 6 μ. 700 ×.

plane structure of the bird retina, I have examined the retina of *Parus major* as regards the presence of visual cell patterns.

METHODS

The eyes were fixed in Bouin's and Fleming's fluids, and their anterior parts were immediately cut away to permit the free entrance of the fixation fluid. Small pieces were then cut out from the retina, their origin was mapped, and each piece (2—3 mm^2 in size) was cut tangentially. The sections were stained in Regaud's haematoxylin.

RESULTS

The structure of the double cones in the bird retina is seen in Fig. 2. The great paraboloid of the accessory cone distends the chief cone, and the nucleus of the accessory cone is situated nearer the external limiting membrane than is that of the chief cone. Fig. 3 shows a tangential section of the retina of *Parus major* on the level A—A of Fig. 2. In this photo the cone pattern can be seen, consisting of groups each composed of one single cone surrounded by

four double cones. The outlines of one such group are traced in the photo. On this level the chief cone is nearly as thick as the accessory cone, but nearly all the chief cones still appear crescent-shaped. Between the distinct groups rods and single cones are found. Moreover, it can be seen (even in the limited part of the retina shown in the photo) that the groups form distinct rows.

Another tangential section, through the cone nuclei in the outer nuclear layer, is shown in Fig. 4, and in this layer as well it is possible to discern the same pattern. However, owing to the increasing number of cones nearer the fovea, there is not room for all the nuclei in the same plane. Owing to this fact they are often situated on different levels, and a section through this layer does not always show complete patterns. The characteristic shape of the double cone nuclei—like an "8" with a small upper part—is due to the unequal size of the two nuclei and their position in relation to each other (cf. Fig. 2).

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DISCUSSION

The pattern found—four double cones forming a square, and a single cone in the middle of this—resembles fundamentally those patterns most common among teleosts, viz. the patterns *D* and *G* of Fig. 1. As the single cones, which are situated in the corners of the pattern *G*, are not regularly found in this position in the *Parus* retina, the pattern mostly corresponds to *D*. However, the fish and bird patterns also differ in the following respect: in the fish pattern the groups may overlap, so that, for instance, the left double cone of one group may at the same time be the right double cone in the group to the left. In the *Parus* retinae examined double cones common to two groups have only been observed in the points of ramifications in the rows, where some irregularities in the general pattern may be seen.

The distinct cone patterns in the retina of many animals have been actualized by the electrophysiological work on the visual cells of teleosts carried out by SVAETICHIN (1956). He found only three types of spectral response curves from the cones: one type in which the two maxima appear in the red and green regions of the spectrum, one with the maxima in the yellow and blue regions, and a third type with one single mean maximum at the wave-length of 574 mμ. Correlating these results with the presence of the double and single cone pattern in the retina of most teleost fishes, he assumes that the colour vision mechanism is found in the double cones, and the general light-sensitivity in the single cones. In the square pattern, the two opposite double cones will, he assumes, represent the same complementary colour pair, and the single cone in the centre will represent the general light-sensitivity.

The presence of the same type of cone pattern in the retina of the Great Tit —which, like most diurnal birds, can be assumed to possess colour vision—

indicates the possibility of using the anatomical structure of the bird retina to explain the colour vision mechanism of birds in the same manner. However, only one species has been examined as yet, and therefore nothing can be said at present about the general applicability of this theory. Still more species, representing different habits as regards diurnal or nocturnal life and different degrees of colour vision, will have to be examined, a work upon which I am just now engaged.

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STUDIES ON THE SUBCOMMISSURAL ORGAN

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This work has been carried out to augment the existing knowledge of the subcommissural apparatus, especially of some weak points such as the innervation of the organ, the composition of its secretion and the ways in which this is given off. As a conclusion an attempt has been made to present a theory of the function of the organ, based on the most recent research. The work has been carried out with the aid of grants from the Swedish Natural Science Research Council.

THE INNERVATION OF THE SUBCOMMISSURAL ORGAN

For a discussion of the significance of the subcommissural organ, it is of course of great interest to know whether it receives nerve fibres, i.e. whether its activity is subordinated to some other region of the brain. The pineal organ has often been proposed as such a directing organ. DENDY (1907) found pineal nerve fibres entering the subcommissural organ of *Geotria*. TRETJAKOFF

(1915), however, was not able to find these fibres in *Petromyzon*, though he paid special attention to this problem. Quite recently Oksche (1954, 1955, 1956) has reported that the pineal tract of anurans almost exclusively innervates the subcommissural cells, either directly or through a neuron. According to this author the pineal organ is light perceptive and directs the activity of the subcommissural organ by means of the pineal tract. In other species, which have reduced, or totally lack, the frontal subcutaneous gland, the epiphysis has acquired mainly secretory properties and gives off no or only a small pineal tract. If there is such a tract, it is said to direct the subcommissural organ. This is contrary to the results of Holmgren (1917, 1918, 1919[1]), who did not find an innervation of the subcommissural organ but was always able to follow the pineal fibres further ventrally in the brain.

For this reason a detailed fibre analysis seemed to be of interest in a species which possesses pineal tracts but no special differentiations of the pineal organ.

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Material and methods. The material chosen was the primitive fin-pike *Polypterus senegalensis* (and a few specimens of the closely related *Calamoichthys calabaricus*, which proved to be almost identical as regards the structures studied). (Material from the Swedish Gambia Expedition of 1950.) In addition to a number of stained series, several impregnation series were used, viz. 22 series according to Ramon Y. Cajal ("Cajal 11" in Romeis), 12 series according to Bielschowsky (modification of Agduhr 1930), 18 Golgi ("rapid" method), 3 Bodian and 4 Weigert (modification of Sheldon 1912) series.

Observations. The pineal organ of *Polypterus* is rather well developed and although it has a very glandular appearance it may contain sense-cells (Studnička 1905). In the distal part of the pineal stalk two separate pineal tracts are found, a left and a right one. These tracts soon divide and the four pineal tract branches are found separately in all the rest of the stalk. At the base of the stalk the left and right anterior branches turn rostrally in the direction of the habenulae, each branch to the habenula of its side without decussating. There are no signs that fibres or collaterals are given off to the subcommissural epithelium. Also the habenular neurons seem to be independent of pineal innervation because each tract makes a curve in its habenula and can be followed further in its course ventro-caudally, close to the tractus habenulopeduncularis, where it apparently still contains the same number of fibres as it already had in the pineal stalk.

The posterior pineal tracts take a caudal direction from the base of the pineal stalk. They can be followed for a short distance among the basal processes of the subcommissural cells but soon disappear among the fibres of the posterior commissure. No fibres could be seen which ended in the subcommissural epithelium.

It is evident that the pineal tracts in *Polypterus* have nothing to do with the subcommissural organ, but the problem still remains how this organ is innervated, if it is innervated at all. There is no evidence of such an innervation in the investigated material. In one single case a fibre was seen among the subcommissural cells, viz. in one of the Golgi series. It was not possible to trace either the origin or the termination of this tiny fibre, which looked very much like the intra-ependymal nerve-endings described by AGDUHR (1922[1]). In another Golgi series a neuron was situated close to the subcommissural cell layer (cf. BARGMANN and SCHIEBLER 1952), with one process directed dorsad and later on caudad in the direction of *tectum opticum*. It is probable that the subcommissural epithelium and this neuron have no more in common than the position.

Discussion. A nervous control of the subcommissural organ activity operated by the pineal organ cannot be the general rule. Such a functional relationship between the two organs must of course be eliminated in animals which lack pineal organs but nevertheless have subcommissural organs which are quite normal, e.g. myxinoids (STUDNIČKA 1905, HOLMGREN 1919), *Torpedo ocellata* (D'ERCHIA 1896) and crocodilians (SÖRENSEN 1894, VOELTZKOW 1903). The findings of OKSCHE may indicate the existence of a specialization in anurans which have a frontal subcutaneous gland, but cannot be of any general importance for the secretory activity of the subcommissural cells.

It has not been possible to find any fibres directed to the subcommissural cells of *Polypterus*. If a special innervation of the organ exists it is reasonable to believe with MARBURG (1922), FUSE (1936) and BARGMANN and SCHIEBLER (1952) that this is effected by fibres which soon lose their identity among the posterior commissure fibres.

THE SUBCOMMISSURAL SECRETION

It is known from the works of BARGMANN and SCHIEBLER (1952) and WISLOCKI and LEDUC (1952[2]) that an important component of the subcommissural secretion is a complex of a carbohydrate, probably a polysaccharide, with a protein. The polysaccharide is believed to be a mucopolysaccharide without acid groups.

PROTEIN-BOUND SULPHUR

Recent investigations on the Reissner's fibre apparatus are largely based on staining methods (chrome haematoxylin, aldehyde fuchsin), which are certainly very useful and stain the structures in question electively, but must be handled

with care as long as almost nothing is known about the chemical nature of the staining processes. In fact, several other structures in the central nervous system (WISLOCKI and LADMAN 1955) as well as in other parts of the body (E. and B. SCHARRER 1954) stain with the same selectivity with these methods. ADAMS and SLOPER (1956) introduced a method for the hypothalamic neurosecretion which demonstrates exclusively structures which are rich in cysteine and cystine. As it is known that the subcommissural cells contain protein-bound disulfide and thiol groups (WISLOCKI and LEDUC 1954) and the nuclei thiol groups (AFZELIUS and OLSSON 1957) it seemed important to examine the different kinds of fibre apparatus secretion in this respect.

Methods. Acid Alcian Blue was used after performic acid oxidation according to ADAMS and SLOPER. Every second section was stained as a control unoxidized with Alcian Blue, mainly according to STEEDMAN (PEARSE 1954). A red tone on the nuclei with carmalum (MAYER) made the preparations more distinct and made the orientation in the sections easier. This method certainly stained the subcommissural secretion distinctly and selectively but from a cytological point of view the preparations often appeared maltreated, apparently as a result of the rather rough oxidation procedure. Better results were obtained with Astra Blau, Bayer (BAHR, personal communication). In a very acid solution also this stain (3 minutes in 1 % solution at pH 0.2 with HCl) stains sulphonate groups exclusively. Adjacent sections were stained with Astra Blau after oxidation of the thiol and disulfide groups to sulphate (in ¼ % potassium permanganate in ½ % sulphuric acid) and decolorizing in oxalic acid (½ %). Also these preparations were counterstained with carmalum. As a specific histochemical test for thiol groups a modification (BAHR 1957) of the BARRNETT-SELIGMAN procedure was used. In some of the preparations the SS bridges were split to show also the disulfide groups.

The material used was fixed in Bouin, 4 and 20 % formalin and 1 % trichloracetic acid in 80 % alcohol. For comparing purposes several sections were stained with chrome alum haematoxylin (GOMORI 1941) and in some cases with periodic acid—Schiff (PEARSE).

Material. Amphioxus (8 mm—adult) 3 specimens, sag., 4—5 µ. *Myxine* (adult) 2 caudal ends sag., 2 brains transv., 4—6 µ. *Salmo* 2 embryos (21½ and 22 days) sag., 4 µ. *Leuciscus* (adult) brain transv. and caudal end sag., 5 µ. *Triturus* (larva) sag., 5 µ. *Rana* (adult) 2 brains sag., 6 µ. *Bos* (adult) 5 subcommissural organs and adjacent regions transv. and sag., 4—12 µ.

Observations

The infundibular organ. In all the investigated *Amphioxus* series the fibre apparatus reacts strongly with the cysteine and cystine tests. The reacting material in the infundibular cells coincides in every respect with the chrome

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haematoxyphile material described by OLSSON and WINGSTRAND (1954). As mentioned by these authors several peripheral infundibular cell processes contain granules or may even be homogeneously stained for a short distance caudally or ventro-caudally. In one of the specimens a marked positive reaction was observed in the dorsal part of the brain, in the region of *recessus neuroporicus*, that is in the very same place where the authors found chrome haematoxyphile granules. Except for these regions mentioned and a faint reaction on some material in the brain ventricle and the central canal, no other parts of the central nervous system reacts with the Alcian Blue or Astra Blau methods.

The flexural organ. The same stages and species were used as in the first description of this organ (OLSSON 1956). The investigation shows that the chrome haematoxyphile material produced by the cells contains SH and (or) SS groups. The same condition is found in several median floor cells of *rhombencephalon* and the spinal cord. The juvenile subcommissural organs of these stages give identical pictures with the reactions in question here as after chrome haematoxylin staining (OLSSON 1956).

The subcommissural organ. The median and slightly paramedian sections through the *Rana* brains clearly demonstrate several accumulations of cysteine (cystine) when stained with Astra Blau or Alcian Blue. These methods are less sensitive than the Barrnett-Seligman procedure and thus demonstrate the great quantities of these molecules only. Several accumulations of this kind are found, viz. those belonging to the praeoptico-hypophyseus system, including fibre tracts and Herring bodies, and on the other hand the subcommissural organ and Reissner's fibre.

An interesting by-product of the investigation is the fact that in addition to the above-mentioned structures a very strong positive reaction was found in a number of cells situated on *plexus chorioideus*. These cells were identified as the cells first described by KOLMER (1921[1]) as leucocytes, later on by J. ARIËNS KAPPERS (1953, 1956) under the name "epiplexus cells". The nature and significance of the sulphur reactions in these cells need further investigation.

The subcommissural secretion reveals a strong affinity for acid Astra Blau and Alcian Blue after oxidation, but it does not stain at all without oxidation. The Barrnett-Seligman method was clearest in *Bos*, for which animal the description below is valid (Fig. 7). The subcommissural structures react in a strongly positive manner already for the thiol group test and it was not possible to decide whether there was an additional reaction in adjacent sections which had been reduced in order to split the disulfide bridges. The two nucleoli are strongly SH-positive and a dark streak is very often observed between a nucleolus and the apical nucleus pole. In *Bos* as well as in *Leuciscus* all the cyto-

plasm, including both cell processes, is rather dark, but there is a particularly strong reaction of the secretory granules and the material on the cell surfaces.

Reissner's fibre always reacts strongly for acid Astra Blau and Alcian Blue after oxidation, whether it is the product of an infundibular organ (*Amphioxus*), a flexural organ (embryonic *Salmo*) or a subcommissural organ (adult vertebrates). In unoxidized sections the fibres do not react at all and it is only the fact that they may stain faintly with the counterstain carmalum that makes it possible to find them at all in these preparations. The Barrnett-Seligman technique reveals that the fibre reacts strongly for thiol groups. No additional reaction for disulfide groups could be established. The caudal mass of the fibre (Olsson 1955) in the different cases gives the same reactions as the fibre.

Discussion

Wislocki and Leduc (1952[2]) found that the secretory material of the subcommissural cells does not stain metachromatically with toluidine blue. This finding is confirmed and extended by the present author by means of the Alcian Blue and Astra Blau techniques, which show that no part of the fibre apparatus contains sulphate groups unless such groups have been produced by oxidation of thiol (or disulfide) groups.

The same authors (1954) were able to demonstrate that the nuclei and distal surfaces of the subcommissural cells stain deeply and Reissner's fibre faintly with Barrnett and Seligman's test for protein-bound thiol and disulfide groups. In the present work efforts were made to distinguish between these two groups and it was found that the infundibular, flexural and subcommissural secretion is rich in thiol groups. The presence of disulfide groups as well could not be determined owing to the dark reaction already obtained by the thiol groups.

The protein-bound SH groups can, in this case, exist only in the form of the amino acid cysteine. As shown by Afzelius and Olsson (1957) the subcommissural cell nucleoli are very rich in cysteine. This indicates a very high metabolism of the cells and it is possible that the nucleoli are the producers of the cysteine-rich substance. The cytoplasm gives a moderate reaction but the granules stain deeply, which indicates that the cysteine-containing substance is accumulated here. The same granules give positive periodic acid—Schiff reaction and Millon reaction (cf. Bargmann and Schiebler 1952, Wislocki and Leduc 1952[2]) which, in this case, means that they also contain carbohydrate and protein. If the authors referred to are right, and the granules contain mucopolysaccharide-protein complexes, it is possible that the cysteine molecules are situated in the protein component. The identical periodic acid—Schiff and cysteine reactions show that the carbohydrate and cysteine-rich protein are always found together in the secretions of the different fibres.

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THE CARBOHYDRATE

Sialic acid. BLIX (1936) and BLIX et al. (1952) isolated from bovine submandibulary gland mucin a substance called sialic acid. KLENK (1941) found that an important component of gangliosides is an acid, which he called neuraminic acid. According to GOTTSCHALK (1955) the sialic acid is an acetylated derivative of the neuraminic acid. Later on sialic acid has been found in the mucin of other glands as well as in other tissues (WERNER 1953). In the central nervous system it is found in the cytoplasm of nerve cells including their processes, in the neurosecretion of the neurohypophysis and especially in certain pathologic conditions, which involve tissue degeneration (DIEZEL 1957).

Sialic acid is found as a component of protein compounds as well as in lipids and polysaccharides. Its structure (GOTTSCHALK) indicates that it must give a positive periodic acid—Schiff reaction as a carbohydrate, but a special histochemical reaction has been worked out for sialic (neuraminic) acid.

Observations. Several sections from the same *Bos* material used in the previous chapter were treated with BIAL's reagent according to DIEZEL (1955, 1957). The reactions observed were not very distinct and unfortunately it was not possible to obtain the material recommended as a control, viz. brain sections demonstrating amaurotic idiocy. The reactions of the various structures in the sections could, however, be estimated with the erythrocytes and blood serum as controls, which structures are known to be rich in sialic acid. While ordinary ependymal cells had but a faint tone of red, the subcommissural cells were darker with a marked reaction on the cell surfaces (Fig. 1). The granules found in the subcommissural hypendyma (p. 89) stained darker than the surrounding tissues. Reissner's fibre could not be found in these preparations but the secretory material in the ventricle, close to the subcommissural cell surfaces, gave the same reaction as the hypendymal granules.

Discussion. Structures in the subcommissural organ which are known to be rich in subcommissural secretion give the same positive reaction for BIAL's reagent as the erythrocytes and blood serum. If the DIEZEL procedure is a method for demonstrating sialic acid electively the subcommissural secretion must be rich in this substance. The sialic acid may be the only carbohydrate of this secretion, perhaps as a sialic protein, or it may occur together with polysaccharides.

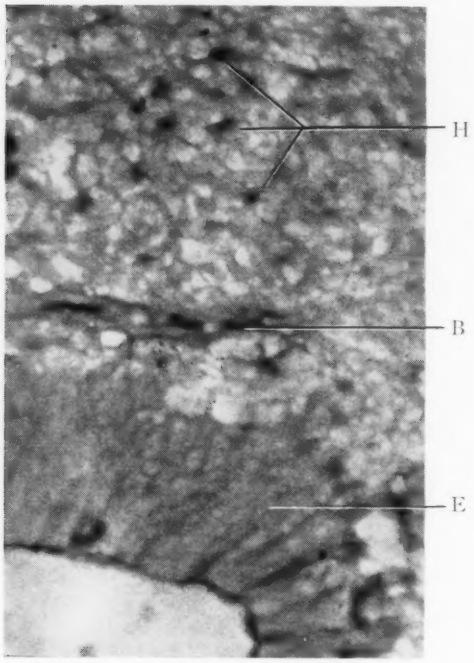

Fig. 1. *Bos*, adult. Subcommissural organ treated with Bial's reagent as a test for sialic acid. Positive reaction is found in the subcommissural ependymal layer (E)—especially at the ventricular border—, in erythrocytes and blood serum (B) and in granule accumulations of the hypendyma (H). (Formol, 10μ, 300 ×. No counterstain. Panchromatic film without filter.)

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CENTRAL RELEASE OF SECRETION

SUBCOMMISSURAL ORGAN AND REISSNER'S FIBRE

The subcommissural organ of Tropidonotus. Most recent authors agree that the subcommissural organ produces a secretory material, which is given off into the brain cavities. Furthermore there can hardly be any doubt that this material, or part of it, forms Reissner's fibre. The relationship between a fibre and an organ in adult vertebrates seems to be established (cf. AGDUHR 1922). BARGMANN (1943), however, has called attention to an exception to this rule. This author was able to picture a normal fibre in the central canal of *Tropidonotus natrix* although REICHOLD (1942) pointed out that this species, as well as *T. tesselata,* belongs to the very few vertebrates which lack a subcommissural organ. Nevertheless WINGSTRAND (1953) presents a picture of this organ in a, probably adult, specimen of *T. natrix*. It is plausible that REICHOLD overlooked the organ in his series and in order to eliminate this problem several transversal series of *T. natrix* (embryos with a head length of 7.6 mm to young animals of

130 mm total length) were studied. All the specimens had subcommissural organs of normal size and form.

Operation of the subcommissural organ. The relationship between fibre and subcommissural organ in old larvas was also demonstrated by electrocoagulation of the organ anlage with the aid of a Hyfrecator (medical instrument from Birtcher Co., Los Angeles, equipped with a finer needle). Several broods of *Triturus punctata* and *T. cristata* were coagulated in the subcommissural region at normal stage 18—19 (WITSCHI 1956). The last treated animal died at n.st. 26 probably because surrounding regions of the brain had also been damaged, as *commissura posterior*. This condition calls for circumspection when the results are judged. However, a fibre was usually found at young stages and it terminated frontally at a flexural organ (OLSSON 1956). In the somewhat older operated specimens the fibre gradually disappeared as a result of the decreasing activity of the flexural organ and the lack of a subcommissural organ, which could function as its predecessor. In one case severe damage in the embryonic fore-brain had produced an "amphioxide" brain type in a larva which lived to n.st. 23. The brain was a single, slightly widened vesicle only, without eye stalks, ventral fold or other differentiations than some chrome haematoxyphile cells in the floor, suggesting an infundibular organ and possibly being the rudiment of the flexural organ. A fibre could not, however, be found in this specimen. It is evident that a normal flexural fibre is produced in *Triturus* embryos in spite of the absence of a subcommissural organ. The fibre disappears when the activity of the flexural organ ceases at older stages. It was not possible to keep such an animal lacking a fibre and subcommissural organ alive for a long period, but this fact does not allow any conclusions about the importance of this apparatus for the animal. It is impossible to destroy the subcommissural organ without damaging other important parts of the brain, especially the posterior commissure, which contains fibres the significance of which is almost unknown to us.

SECRETION IN FIBRE FORM

If the view is accepted that Reissner's fibre is formed of the subcommissural cell secretion, two important questions arise. Firstly, is it possible for a substance released into the cerebrospinal fluid to form this perfect fibre form? Secondly, has the secretion a function which can be maintained in this curious shape only? The last question will be discussed in connection with the function of the organ (p. 97). An unsuccessful attempt to dissect out the fibres of unfixed cow brains, however, gave other results, which seem to answer the first question in the affirmative.

Material. 22 brains of calves and cows (*Bos*), two brains of pig (*Sus*) and a single one of horse (*Equus*) and of sheep (*Ovis*) were used. The subcommis-

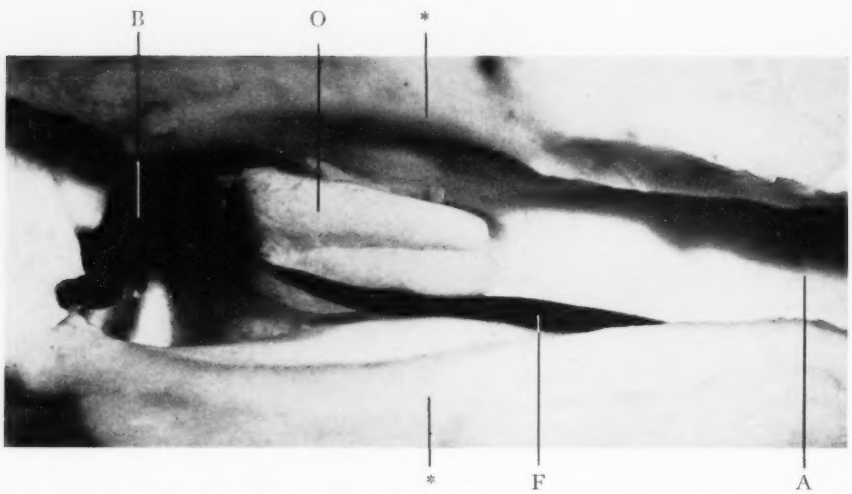

Fig. 2. *Bos*, adult. Ventral view of the subcommissural organ (O). A blood-clot in the third ventricle (B), is caudally continued in the form of a fibre (F), which can be followed behind aquaeductus mesencephali (A). Asterisks indicate orientation of the section pictured in Fig. 6. (10 ×.)

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sural organs were carefully dissected free from the ventral side as quickly as possible, which was usually performed within 15—20 minutes after death. My sincere thanks are due to Mr. N. Hegnell, chief veterinary officer of the Gothenburg abattoir, who placed the material at my disposal and facilitated my work in every respect.

Observations. It was not possible in any single case to dissect out Reissner's fibre in "living" condition although this could be done after fixation of this material as well as that of smaller mammals (*Lepus*). In the cows, which had been killed by shooting, hemorrhage had occurred, with the result that a large blood-clot was usually found in the third ventricle (Fig. 2). In almost all of the investigated cows this blood-clot was caudally extended in the form of a fibre, which could be followed behind the subcommissural organ, through the mesencephalic aquaeduct and, in some cases, in the fourth ventricle.

Discussion. The tendency of the blood, after a bleeding in the rostral part of the brain cavities, to coagulate in the form of a fibre in the caudal brain cavities must be the result of the ventricular fluid movements and the physical properties of the blood. While still flowing, the blood has run caudally as a narrow stream and gradually threads of fibrin have been formed in it and have attracted the blood-cells, which has resulted in this elongated clot. It is true that in these cases the caudal movement of the blood may be the result of pressure changes in the ventricles caused by the damage. But it is well known

that in the living brain the ventricular fluid moves backwards in the ventral parts and forward in the dorsal parts as a result of the ciliary activity (JOHNSTON 1903). The circulation of the liquor has been studied with the aid of pigment particles found in it (WANG and LU 1941, CHU 1942, ADAM 1953) and it is evident that it follows strict courses. The movements of the fluid in *epithalamus* are not known (particles placed close behind the subcommissural organ of the cow were swept forwards), but from the organ Reissner's fibre is directed caudo-ventrally where a linear caudal "Strömungsbahn" (ADAM 1953) flows all the way to the caudal end of the central nervous system. Apparently the physical properties of the fibre material are such as to permit the material to condense in the form of a viscid fibre if only it is prevented from being dispersed, which is effected by the liquor streams. This is best understood in the case of the flexural secretion (OLSSON 1956). This material is delivered in the above-mentioned caudal stream which must be believed to exist already very early during the ontogeny because a ciliated groove is found in the floor of the central canal already in the early development of the neural tube (BEARD 1888). Later on the subcommissural secretion also contributes to the formation of the fibre and perhaps this is not possible until the subcommissural and posterior commissure fold has grown out and caused a whirling motion of the epithalamus liquor and a median stream directed downwards to the mesencephalic floor.

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THE CONSISTENCY OF REISSNER'S FIBRE

In the brain, close to the subcommissural (infundibular, flexural) organ, the fibre is divided into a great number of tiny fibrils which caudally fuse and form Reissner's fibre: "—es scheint zu einer wirklichen Substansverschmelzung der Teilfäden zu kommen, so, wie sie etwa bei miteinander verschmolzenden Glasfäden beobachtet wird—"(KOLMER 1921[2], p. 699). As it now seems unreasonable to believe in such a static condition of the fibre, but rather that it moves continuously backwards in the cerebral cavities (OLSSON 1957), the consistency of the fibre substance must be such as to permit the tiny fibrils to fuse homogeneously. The fact, however, that EBERL-ROTHE (1951) reported success in dissecting fresh fibres out of the brains of some mammals and could handle them with forceps, does not speak in favour of such a viscid state of the fibre substance. In order to obtain information about what happens when two Reissner's fibres meet in the central canal, some double-headed young salmon specimens were examined.

Material. From a collection of young *Salmo salar* specimens with *duplicitas anterior,* three animals were chosen, about 20 mm in length, with the duplication extending for varying distances. The heads and the tail of each animal were cut in sagittal sections (7 μ) while the place of junction was cut horizontally

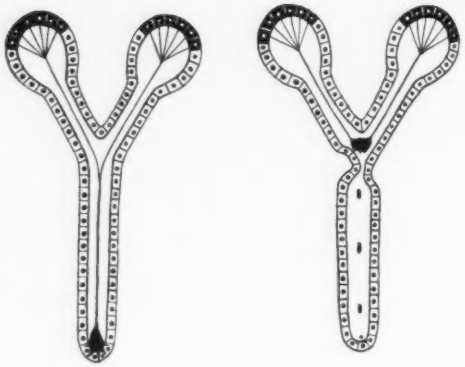

Fig. 3. Diagrams illustrating the appearance of the fibre apparatus in two cases of duplicitas anterior in Salmo. Subcommissural cells black with white nuclei, fibre material black.

and stained with chrome haematoxylin (Gomori 1941). The material was kindly placed at my disposal by Mr. N. G. Steffner Jr., Älvkarleby hatchery.

Observations. One specimen had a severe defect in the spinal cord close behind the junction, which had resulted in a partly blocked central canal in the anterior part of the tail (Fig. 3, right). The heads normally produce fibres which, however, lose their thread-like form in front of the closure of the central canal and terminate as a common caudal mass at this place. Behind the closure the central canal is normal again, but no fibre can be found here, only small accumulations of chrome haematoxyphile substance, which indicates that small amounts of fibre substance may pass the closure.

In the other two specimens the central canals are quite normal with the exception of the duplications. At the junction, the two Reissner's fibres come into proximity, parallel each other for a short distance in the common central canal and finally fuse and form a single and somewhat thicker fibre (Figs. 4 and 3 left). The caudal termination of this unpaired fibre is a very well developed caudal mass in the caudal end of *filum terminale*. One of the examined specimens was used for measurements of the fibre sizes. The two paired fibres had not the same diameter and a series of measurements revealed for the thinner one a mean diameter of 0.7 μ, for the thicker one a diameter of 0.9 μ and for the unpaired fibre behind the junction a diameter of 1.2 μ. This means that the sum of the transverse areas of the two paired fibres is 1.0 μ^2, the same area of the unpaired fibre being 1.1 μ^2. Also having regard to all possible errors due to the measurements and the possibility that the fibre is not perfectly rounded at all places measured, this indicates that the caudal, single, fibre contains approximately the same amount of substance per unit of length as the two paired fibres together. This is quite in accordance with the opinion that the fibre is continually moving backwards in the brain cavities (cf. Olsson 1957.)

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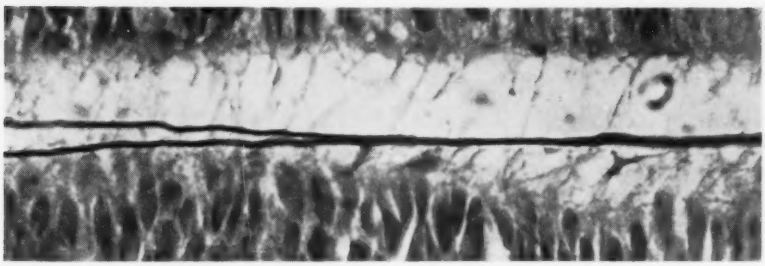

Fig. 4. *Salmo*, 20 mm, specimen with duplicitas anterior. Horizontal section through the place of fibre fusion. (5μ, Cr.-Htx., 750 ×.)

Discussion. Evidently the consistency of Reissner's fibre, in spite of its perfect thread-like form, is not more solid than that it permits a uniform confluence of two fibres. Thus there seem to be no difficulties in believing that the fibre is incessantly formed in the brain by the fusion of innumerable tiny fibrils, in about the same way as the silk threads leaving the spinneret.

THE CAUDAL END OF THE FIBRE

In one of the double-headed salmon (Fig. 3, right) it was noted that the fibre form is lost when the central canal is partly blocked, although some substance may pass the obstacle. This might be explained as the result of disturbed liquor movements. The same appearance of fibre termination is however found in the caudal end of a normal spinal cord (Olsson 1955) as well as in front of a breakage of the spinal cord (Olsson 1957). This caudal mass gives, in sections, the impression of being less viscid than the fibre and it is astonishing that it does not follow the dorsal liquor streams back to the brain again. As the caudal mass appears to be the only part of the fibre apparatus which can be studied in vivo it seemed important to control the results obtained from histological sections by this means.

Material and methods. Newly hatched *Leuciscus* fry proved to be transparent and usually pigment-free enough to permit a microscopical investigation of the terminal ventricle. The body of the fish was kept on an object glass in a small plasticine holder containing water with the protruding tail covered with a cover glass. A magnification up to 600 × was possible with this method. A new animal was used as soon as the erythrocytes tended to slow up their speed in the smallest capillaries. Phase contrast and ordinary light microscopy were used.

Observations. As could be expected it was never possible to find Reissner's fibre in the living specimens. But the action of the cilia in the spinal cord was very clearly demonstrated by the turbulence of the central canal material. In

some cases the action of the tall ventral cell cilia was easily seen. From an erect position they made a strong stroke in the caudal direction, which means that the central canal material was forced backwards. In the terminal ventricle a strongly light refractive mass was always found, which apparently had a fixed position but incessantly made trembling movements owing to the turbulence of the liquor. With the aid of protrusions the mass often communicated with the ependymal cells, between which droplets of the same material could be seen. Similar droplets could also be seen in the mesenchyma outside the spinal cord. If the mass was carefully watched for a while it was found that it slowly changed its shape. In some cases it was possible to see mass protrusions give off droplets among the ependymal cells and also droplets that entered the mesenchyma from the spinal cord cell layer. One specimen, which had been carefully pictured as regards the position of this refringent material, was used as a control. It was rapidly fixed and the tail was cut sagittally on the microtome and stained with chrome haematoxylin. The material observed in the living specimen stained intensely and proved to be identical with the "caudal mass" of Reissner's fibre.

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Discussion. The caudal mass has a gluey consistency and gives off material which penetrates between the ependymal cells. It is also evident that the viscid fibre material is well preserved by the fixing fluids, which means that the preparations of this structure may be considered representative.

PERIPHERAL RELEASE OF SECRETION

PRIMITIVE FIBRE-FORMING CELLS

The flexural and primitive subcommissural cells preserve their spongioblastic character and have a ventricular position but reach the outer limiting membrane with the peripheral processes (Figs. 5 and 9 A). Secretory material may be found in these cells basally as well as apically to the nucleus but there are no indications of a secretory release in any other direction than centrally, that is into the ventricle (Olsson 1956). The basal secretory granules may be looked upon as a kind of intracellular store in the same way as Hild (1951) considered granules found in the dendrites of nerve cells to be stored material, which was later released via the axons.

THE SUBCOMMISSURAL CELLS IN HIGHER VERTEBRATES

In higher vertebrates the subcommissural cells may lose their footing on the limiting membrane and terminate in the nervous tissue. Thus Oksche (1956), already in anurans, found subcommissural cells terminating at blood vessels and suggested a secretory release this way. The same condition was reported

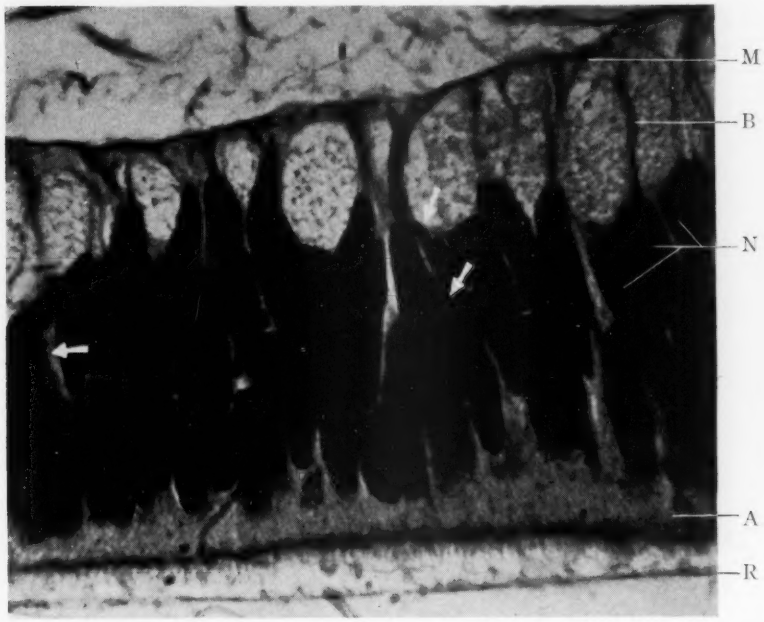

Fig. 5. *Salmo*, 75 mm. Primitive, spongioblastic subcommissural cells. M. Membrana limitans gliae externa. B. Basal and A. apical cell processes. N. Nuclei. R. Reissner's fibre. Note chrome haematoxyphile granules in the basal processes (arrows) and strong haematoxyphily in apical processes. (Sag. 4μ, Cr.-Htx., 1,500 ×.)

from mammals by BARGMANN and SCHIEBLER (1952). WISLOCKI and LEDUC (1952[2]) on the other hand denied a close contact between the subcommissural cells and the capillaries. The authors found that each capillary in this region is "surrounded by some kind of a sheath which separates it from immediate contact with the nearby epithelial cells" (p. 524).

With the intention of studying these structures the subcommissural organ of the cow was chosen because this animal is fairly well known as regards the development (TURKEWITSCH 1936[1,2]) and the anatomy (BAUR-JOKL 1917, KRABBE 1925) of the subcommissural organ and the development of Reissner's fibre termination (AGDUHR 1922[2]).

Material. The subcommissural organs from four cows, fixed in formalin and Bouin's solution, were cut in different orientations in 4—12 μ sections, which were treated with azan (HEIDENHAIN) chrome haematoxylin (GOMORI), Astra Blau (see p. 74) with and without previous oxidation ($KMnO_4$) and with the test for protein-bound sulfhydryl and thiol groups according to BARRNETT and SELIGMAN slightly modified according to BAHR (1957).

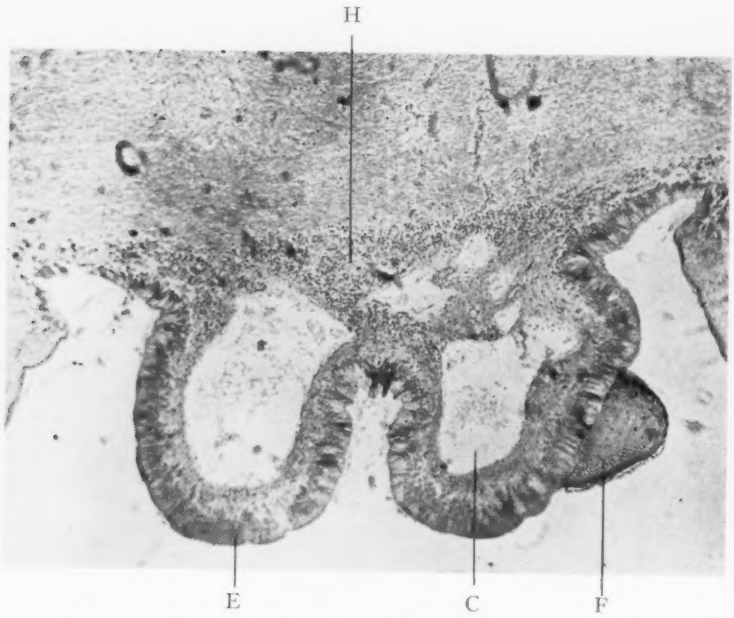

Fig. 6. *Bos*, adult. Transverse section of the subcommissural organ pictured in Fig. 2, at the level marked with asterisks. F. Blood-clot. E. Fold of the ependymal layer. H. Hypendymal layer with granule accumulations appearing as black dots. C. Hypendymal cavity. (6μ, Cr.-Htx., 41 ×.)

Observations. The ependymal layer of the subcommissural organ of the cow is very suggestive of that of carnivores, as described by BARGMANN and SCHIEBLER. Its surface lining the ventricle has been greatly enlarged in two ways. Firstly the organ forms two large folds hanging down in the ventricle (Fig. 6) which, especially caudally, are of imposing size. Furthermore the epithelium is, especially frontally, richly invaginated forming a number of crypts and canals in the hypendyma.

Reissner's fibre is very thick in the cow (14 μ in diameter according to AGDUHR 1922[2]) and some of its fine terminal fibrillae can be seen close to the subcommissural epithelium. It is, however, possible to trace the fine fibrillae to the median portion of the organ only, although the other parts of the organ also evidently have secretory properties as well. In fact it often seems as if the secretory activity in the lateral pockets and the canals is especially high.

The basal processes of the ependymal cells are rather short and can easily be seen, especially in the Barrnett-Seligman preparations, where they are seen to terminate with broadened feet at the *membrana limitans gliae perivascularis* of the hypendymal capillaries (Fig. 7). The layer of ependymal feet seemingly

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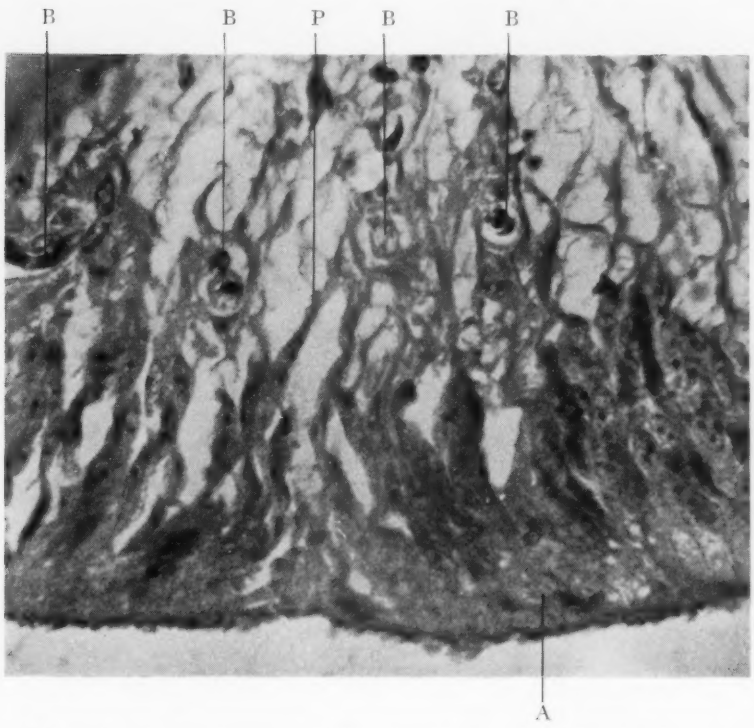

Fig. 7. *Bos*, adult. Subcommissural ependymal cells; SH and SS group reaction. Note the dark reaction of nucleoli, apical cell processes (A) and basal cell processes (P), which terminate at the membrana limitans gliae perivascularis round the blood vessels (B). (6μ, Barrnett—Seligman technique, 750 ×.)

forms a thick sheath round the vessel. It was not possible to decide whether the fairly large space between the glial layer and the endothelium represents a Virchow-Robin's space or should be interpreted as an artifact.

Discussion. All the subcommissural cells of the ependymal layer of the cow appear to give off secretory material into the ventricle. It seems reasonable to believe that all the cells contribute to the formation of Reissner's fibre (Wingstrand 1953) although it may seem as though the fibre is connected to only part of the organ (Bargmann and Schiebler 1952). In fact, each of the invaginated tubules of the subcommissural epithelium of the cow is reminiscent of the subcommissural canal of *Myxine* (Afzelius and Olsson 1957). Also in this case the secretion at the beginning mainly forms an amorphous mass in the canal lumen, which only gradually condenses to fibre form.

The staining reactions show that the basal processes may contain subcommissural secretion as well as the apical ones. No accumulation of granules in

the ependymal feet or other indication of a peripheral release has, however, been found. But it must be noted that the anatomical prerequisites for a delivcrance to the capillaries exist. The protecting sheaths round the vessels, described by WISLOCKI and LEDUC (1952[2]) in some rodents, are, unless identical with the ependymal feet layer, not found in the cow.

THE HYPENDYMAL LAYER

Hypendymal secretion. The subcommissural organ of higher vertebrates consists of two clearly distinguishable parts, i.e. *the ependymal layer* and *the hypendymal layer*. The first-mentioned layer is the original part of the organ, the modified ependymal cells lining the ventricle. In mammals a loose glial tissue, which KRABBE (1925) named the hypendyma, develops beneath this layer.

Even in fishes it is rarely possible to find granules in cells situated beneath the subcommissural ependyma, but it is not until the mammal level is reached that this condition seems to be more general. WISLOCKI and LEDUC (1952[1,2], 1954) thus describe in the rat (*Rattus*) so-called periventricular glial cells ("presumably microglia"), which have a subependymal situation but are widely distributed in the central nervous system as well. In the guinea pig (*Cavia*) these authors found the same kind of glial cells especially crowded beneath the subcommissural epithelium. BARGMANN and SCHIEBLER (1952) found irregular complexes of granulated glial cells in the hypendyma of the dog (*Canis*) and the cat (*Felis*), the granules of which have the same staining properties as those of the ependymal layer.

The hypendymal layer of the cow. The same cow material as that mentioned above was used for an investigation of the hypendyma of the cow. The hypendymal layer consists of a very loose, fibrillar glial tissue with but few nuclei. In the two ventricular folds there are hypendymal cavities, which do not communicate with the ventricle, lack an epithelial lining and are occasionally traversed by hypendymal bridges (Fig. 6). Several glial fibres are found in this region, apparently the processes of fibrillar astrocytes. These fibres are very often found deeply in the ependymal layer. A striking feature of the hypendyma are the several cellular islands (apparently the same as those mentioned by KRABBE 1925), which are usually found close beneath the ependymal layer. Similar islands are also often found interspersed among the fibres of the posterior commissure and even as dorsally as in the ventralmost portion of the pineal gland.

The basic structure of these islands seems to be a ball of cells, with the nuclei situated centrifugally and a small cavity in the centre. The reason for believing this is the fact that a picture like that in Fig. 8 is always obtained in sections, independently of the orientation of the section. The nuclei of these

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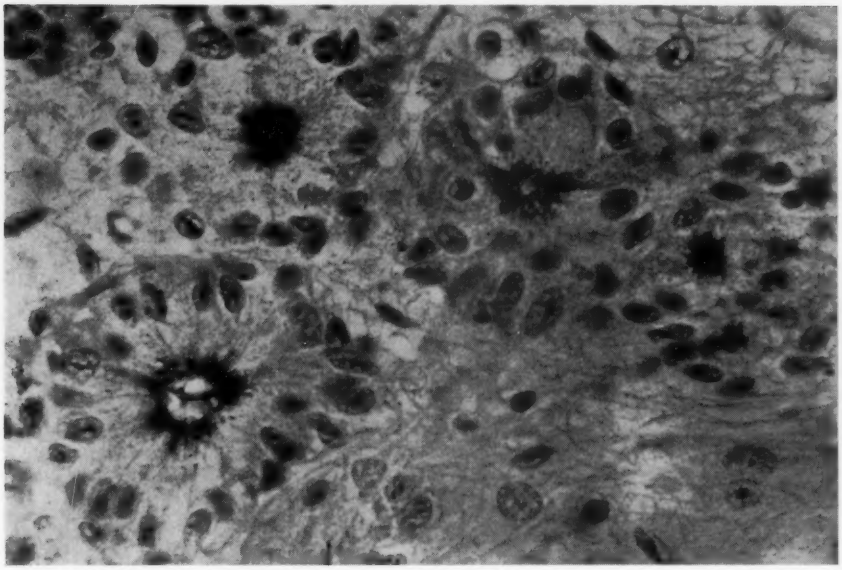

Fig. 8. *Bos*, adult. Hypendymal rosettes (cf. region H. in Fig. 6). At bottom left a rosette which has been cut through the great circle, showing granulated central cell processes and secretory material in the cavity. (10μ, Cr.-Htx., 640 ×.)

cells are very like those of the ependymal layer and they also frequently contain "nucleolar streets" (emissions) between the nucleoli and the nuclear membranes, which indicates an active protein synthesis in these cells as well. The nuclei are situated basally, leaving a rather large apical cell process free. Here a number of granules are found, crowded close to the small cavity but also forming rows of granules in the direction of the nucleus. Often, but not always, an accumulation of an amorphous material is seen in the central cavity. This material, as well as the cytoplasmic granules, stains intensely with chrome haematoxylin, acid Astra Blau after previous oxidation and reacts positively for the sulfhydryl group test. It is not stainable with Azan or Astra Blau when unoxidized, that is, it shows the same reactions as the similar granules and material produced by the ependymal layer. No differences in size of the hypendymal cells could be seen, which might indicate different phases of activity, nor was it possible to find mitoses as signs of old cells being replaced by young ones.

It is extremely difficult to obtain a clear picture of the fate of the peripheral processes of the island cells, probably because of the fact that several processes intermingle and their courses are far from straight. Furthermore the picture is often confused by narrow bridges which traverse the glial tissue and connect two islands or an island and the ependymal layer. These bridges contain cells with extremely elongated nuclei. Also in this case the sulfhydryl reaction pre-

parations are the most useful ones and in these it seems evident that at least a number of the basal processes of the island cells terminate at blood vessels in the same manner as those of the ependymal layer. The hypendyma is fairly well vascularized and in a few cases it was possible in oxidized preparations stained with acid Astra Blau to find dark blue droplets in the blood vessels of this region.

Origin of the granulated hypendymal cells. It must be considered very uncertain whether the granulated hypendymal cells in some rodents, for which WISLOCKI and LEDUC (1954) proposed a microglial nature, can be homologized with the hypendymal island cells of the cow, partly because of the wide-spread distribution of the afore-mentioned cells in some animals, partly because of the fact that nothing is known as regards their origin. The irregular glial complexes, however, which BARGMANN and SCHIEBLER (1952) found in some carnivores, show every sign of being identical with these islands of the cow. The investigations of KRABBE (1925) make it likely that a similar hypendyma may be found also in pinnepedias, cetaceans and primates.

The ontogenetic investigations of TURKEWITSCH (1936[1]) throw some light upon the nature of these islands. In the calf (*Bos*) the hypendyma is first seen in an embryo 100 mm in length and it is developed from the ependymal layer. During the development the hypendyma becomes increasingly loose but the original compact tissue remains in the form of scattered islands. There can hardly be any doubt that these islands mentioned by TURKEWITSCH are identical with those studied in this paper. This ontogenetic investigation as well as the close similarity in their cytology makes it reasonable to interpret the hypendymal island cells as ependymal cells which have invaginated and lost their contact with the ventricular surface.

Most of the islands are found close beneath the ventricular epithelium but the fact that they are also found in abundance among the fibres of the posterior commissure and in the proximal and posterior part of the pineal gland, makes it necessary to take also the pineal gland ependyma into consideration.

Pineal gland ependyma. The occurrence of ependymal cells in the mammalian pineal gland has often been reported. Thus DIMITROWA (1901) described in *Bos, Ovis* and *Canis* small cavities "which resemble thyroid vesicles or the anterior pituitary lobe" (TILNEY and WARREN 1919). The same structures were observed in *Bos* by ILLING (1910) and in different mammals by CUTORE (1910), who described them as epithelial cells containing granules and delimiting the cavities of tubules or acini. COLLIER (1944 p. 66) investigated rodents and found "—von Gerinnseln ausgefüllte Hohlraumbildungen vom Typys der Ependymrosetten—". FUNKQUIST (1912) described these so-called "ependymal rosettes" in a 480 mm long *Bos* and reported that the cells have long, slender

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basal processes and that apically they send cilia into the central cavity. The occurrence of cilia was later denied by BARGMANN (1943). Similarities between ependymal elements of the pineal gland and of the subcommissural organ have been reported by KRABBE (1916, 1925), CAPURRO (1942) and CANEPA (1949).

The ependymal rosettes of the pineal gland, as well as those of the subcommissural organ, must be considered to be the remains or the descendants of the primitive epithelial lining of the ventricle. The question then arises from what kind of epithelium they are derived, what potencies they may carry. The *Bos* material investigated indicates that a number of the pineal rosettes are of subcommissural origin and still contain granules of the "subcommissural secretion type". These granulated rosettes are found in the caudal and ventral portion of the pineal gland of *Bos*. It is very likely that also the human pineal gland has incorporated subcommissural material. BARGMANN (1943) states that the posterior lobe of the human pineal gland receives a secondary invasion of ependymal material during the ontogeny. Furthermore the same author found the ependymal rosettes of *Homo* to be most abundant "in der Grenzzone zwischen hinterem Umfang der Zirbel und der zur Commissura caudalis überleitenden Lamelle —" (p. 401). TURKEWITSCH (1936[2]) directly homologized the subcommissural hypendyma of *Bos* and *Ovis* with the posterior pineal gland lobe of *Homo*.

The investigated *Bos* material showed that besides the granulated rosettes, several other rosettes were found with an identical appearance but with the single exception that they lack the cytoplasmic granulation. Because the granulated rosettes, as stated above, are always found in a limited region of the pineal gland, while the others may be found anywhere, it seems unreasonable to interpret the two as only different states of function. It is more reasonable to look upon the non-granulated rosettes as remnants of an ependyma without the potency of forming the "subcommissural secretion".

Significance of the granulated rosettes. The subcommissural cells of the ependymal layer are indubitably very active glandular elements, the synthesized material of which may be found as granules of different sizes apically as well as basally to the nucleus. There seems to be no doubt whatever that a secretory release takes place from these cells into the ventricle. In higher vertebrates the basal processes are found terminating at blood vessels. In these cases also a basal release of secretory material is possible, as suggested by the staining reactions of these processes (cf. Fig. 7).

The hypendymal island cells apparently maintain most of the features of the ependymal cells with the single but important exception that they do not reach the ventricle. The nucleolar apparatus still indicates an intense protein synthesis, the cytoplasm still contains granules with the very distinctive histochemical characters of the subcommissural secretion and the basal processes apparently

still reach blood vessels. Unquestionably also these cells produce the granules which in this case are released into the small central cavity. If this is true, it is tempting to look upon the hypendymal rosettes as a "store gland" like the thyroid gland (lit. in BARGMANN 1939). The apical secretion thus forms a store ("colloid") in the central cavity, which may be resorbed and in the form of a basal secretion delivered to the blood vessels. Also a direct basal release is of course possible in this case as well but has not been established.

CONCLUSIONS

THE PHYLOGENY OF THE SUBCOMMISSURAL CELLS

In 1938 TILNEY drew attention to the marked difference in phyletic constancy between the roof glands and the floor glands of the vertebrate fore- and interbrain. While the floor glands are pronouncedly constant throughout the vertebrate series the roof glands are extremely variable and inconstant. TILNEY did not take into consideration the subcommissural organ, which at that time was but poorly known. This organ, however, appears among the other derivatives of the roof-plate as a structure of remarkable phyletic constancy. It is found with only slight variations in all the vertebrate classes and it seems as though a structure with the same texture, and possibly with the same function, has been acquired very early in the development of the chordate phylum (OLSSON and WINGSTRAND 1954, OLSSON 1956). This structure is a gland which produces secretory material into the brain cavity, where the material takes the form of a fibre (Fig. 9 A). A slight modification of the structure is found at a higher level in the vertebrate series (Fig. 9 B). The gland cells have now changed their basal termination from the outer limiting membrane to the blood vessels, which may be a consequence of the increasing distance between the inner and the outer membrane with the growth of the brain. Furthermore there may be a tendency in some mammals to enlarge the ventricular surface of the organ by means of folding and invaginations (Fig. 9 C). Only quite recently—in the time-scale of chordate evolution—a differentiation of the subcommissural organ has occurred, namely in the higher mammals. And this differentiation comprehends degeneration as well as specialization. The *degeneration* concerns the original fibre apparatus which is rudimentary or totally lacking in several species of different mammalian orders (see KRABBE 1925, BARGMANN 1943): the insectivores (*Sorex*), the cetaceans, the anthropoid apes and man. The *specialization* of the subcommissural ependyma means that part of it has been withdrawn from its ventricular position deeper into the nervous tissue (Fig. 9 D), where the cells still continue to produce subcommissural secretion although this cannot possibly now form a fibre in the ventricle.

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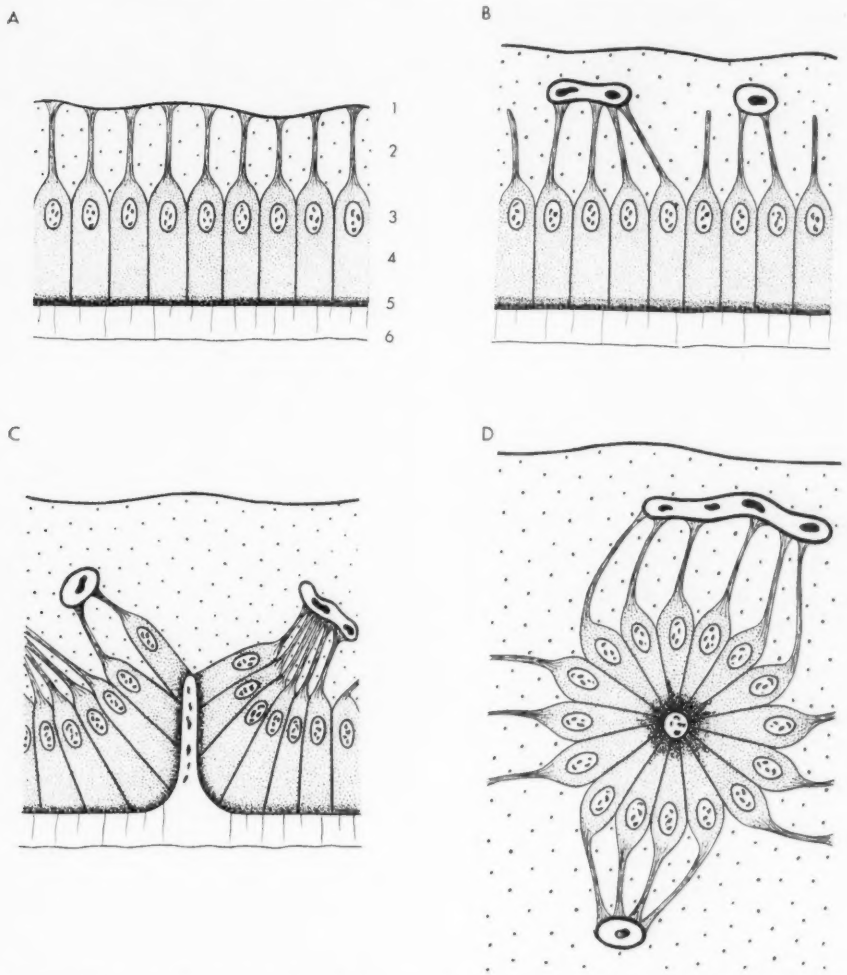

Fig. 9. Diagram illustrating the phylogeny of subcommissural cells. *A:* Primitive cells which reach the external limiting membrane (*1*) with the basal processes (*2*). Below the nucleus layer (3), the apical processes (4), lining the ventricle (5), in which Reissner's fibre (6) is situated. (Cf. Fig. 5.) *B:* Advanced cells basally terminating at blood vessels (cf. Fig. 7). *C:* Modification with ependymal invaginations into the hypendyma. *D:* Hypendymal rosette composed of cells reaching neither the external limiting membrane nor the ventricle (cf. Fig. 8).

THE FUNCTION OF THE FIBRE APPARATUS

Must the fibre necessarily have a function? Some authors have proposed that the fibre apparatus has no special purpose for the animal (JORDAN 1919). Later on C. U. ARIËNS KAPPERS (1947 p. 74), writing about the fibre, said: "Il semble, toutefois, qu'elle n'exerce aucune influence spéciale." It seems un-

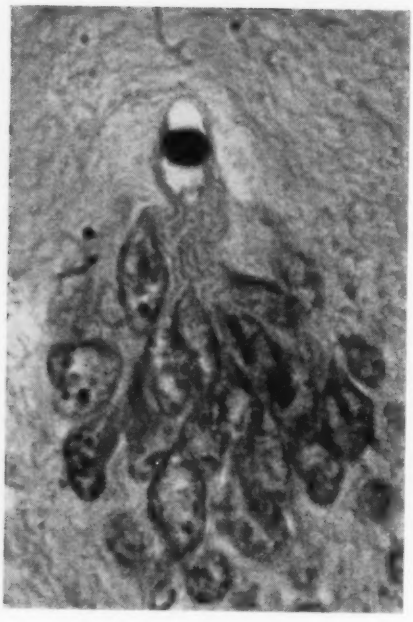

Fig. 10. *Myxine*, adult. Reissner's fibre (as a black circle) passes the nervous tissue of the mesencephalic region through a diminutive canal. The ependymal cells of this canal are clustered as a cord of cells below the canal. Note their ventricular processes between the canal and the nuclei. (Transv. 5 μ, Cr.-Htx., 1,400 ×.)

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likely, however, that the fibre apparatus is a structure without importance, perhaps a mere vestige of a once important mechanism. Already the remarkable phylogenetic constancy speaks in favour of the opinion that the fibre apparatus, as well as for instance the hypophysis, constitutes an integral part of the vertebrate brain. Also from the ontogeny an example may be mentioned, which indicates how important it is for the animal to preserve the fibre apparatus.

The brain cavities of *Myxine* are greatly reduced during the development (Holmgren 1919[2], 1946, Jansen 1930). In the mesencephalic region the fibre passes through a mass of nervous tissue by means of a diminutive channel (the "isthmic" canal according to Nicholls 1912) which gives the strong impression of having but a single purpose: viz. to allow the passage of Reissner's fibre in its usual course (Fig. 10). Also the fact that the fibre apparatus is lacking in some mammalian species speaks against the opinion that the fibre is a most conservative vestige, which has been nothing but a burden for the vertebrate stem for a very long time. When it is no longer needed, or when its function has been replaced by the function of some other structure, it may disappear quickly during the evolution, and can be recognized as a fetal rudiment only (Dendy and Nicholls 1910, Kolmer 1921[2], Krabbe 1925, Keene and Hewer 1935).

Earlier theories of the function. Many theories have been propounded to explain the function of the fibre apparatus, but only one of them has been able to exist for a while, viz. that of DENDY (1909) with several later modifications, which maintains that the fibre is part of an intracerebral sense organ (literature and short survey of earlier theories in WINGSTRAND 1953). Most recent authors, however, seem to agree that the function of the subcommissural organ is to produce active principles into the ventricle (or elsewhere).

GILBERT (1956) believed that the organ produces a hormone regulating water uptake. The experiments need confirmation and do not seem to be in accordance with the results of WINGSTRAND 1953. FRIDBERG and OLSSON (not yet published) have studied the praeopticus—hypophyseus system in fishes which were suddenly moved from fresh to salt water and vice versa. Although a marked response could be observed in this system, the sudden change in the water balance did not affect the subcommissural secretion.

OKSCHE (1957) believed in the release of a principle regulating the colour-change in anurans. It has previously been pointed out that these results may not be generalized and cannot explain the constancy of the fibre apparatus. It must also be remembered that a response of the secretion to the intensity of light has been observed also in other cases, e.g. in the hypophysis (MILIN and CIGLAR 1957). ADAM (1957) proposed that the subcommissural organ of *Myxine* produces spinal fluid. This proposal is apparently based on two facts only: *Myxine* lacks a chorioid plexus—which is usually believed to produce this fluid—and the subcommissural organ is very well developed in this species. The possibility of the subcommissural cells being able to replace the plexus cells seems, however, very remote because of the great differences in cytology of the two types (AFZELIUS and OLSSON 1957) and the difference between the substances synthesized in the subcommissural cells and those found in the cerebrospinal fluid. Furthermore it seems unlikely that the organ of *Myxine* should have a function different from that of the almost identical organs found in other vertebrates.

Reissner's fibre and the cerebrospinal fluid metabolism. There can hardly be any doubt that the cerebrospinal fluid is of great importance for the metabolism of the nervous tissue. The liquor—brain barrier (WALTHER 1933) seems to be easier to pass than the blood—brain barrier and if a stain is introduced into the cerebrospinal fluid, it will penetrate the nervous tissue according to the laws of diffusion (SPATZ 1933) if the particles do not exceed a certain size (BAKAY 1956). As a matter of fact the liquor must contain also waste products from the nervous tissue (HASSIN 1948) and it is likely that a high concentration of this waste must be fatal because the nervous tissue is not capable of preventing the toxic substances from penetrating the tissue. The importance of the liquor composition is also demonstrated by the fact that a

weak dose of a toxic substance, which produces no effect when injected into the brain arteries, may prove lethal when introduced into the cerebrospinal fluid (GOLDMANN 1913). It is with the aid of this knowledge about the importance of the cerebrospinal fluid that we have, I feel, to search for the function of Reissner's fibre. Three substances have been reported as being components of the fibre substance, all of which may play an important role in liquor reactions, viz. cysteine, mucopolysaccharides and sialic acid.

Cysteine is of importance for a great number of enzyme activities and the rich supply of this amino acid may indicate that the fibre participates in the metabolism by releasing and receiving substances important for the enzyme processes in the nervous tissue. It must be noted that small accumulations of cysteine-rich granules have in some instances been found at the ventricular surfaces of the spinal cord ependymal cells. Cysteine may also have a biological importance as detoxicating agent, depending on the importance of SH groups in connection with intoxications (lit. in BAHR 1957).

Mucopolysaccharides. It is likely that the fibre substance contains mucopolysaccharides and it is now known that these large molecules in other tissues have a far more important function than that of acting merely as a support. They play an important role in the ground-substance of the connective tissue by regulating the water and electrolyte balance and it is known that each molecule has a limited "life" depending on the activity level of the tissue in question (WYBURN and BACSICH 1957). The mucopolysaccharide molecules of the connective tissue ground-substance are broken down by enzyme activity when they have served their time and this is perhaps what happens to the fibre substance when it becomes invisible by histological means in the caudal end outside the central nervous system.

Sialic acid may be present in the fibre, which seems likely with the histochemical methods available today. Very little is known about this substance but it is probable that it may act as a cell receptor: —„ sie ist wohl das hypothetische Mucoproteid, welches nach BURNET die Haftung eines Virus an die Zelle ermöglicht (KLENK und STOFFEL)" (DIEZEL 1957, p. 3). And about the importance of sialic acid the same author writes (p. 73): —„sprechen unsere Befunde für ihre Beziehungen zu den Zellreceptoren, ist sie doch überall da vermehrt, wo im gestörten Zellstoffwechsel Produkte enstehen, die von der Zelle ‚unschädlich' gemacht werden müssen."

All these substances, or at least two of them (p. 77), are constituents of the subcommissural secretion and it is evident that their biological importance can be twofold. They may act as principles actively taking part in the metabolism of the liquor material and they may act as detoxicators, which keep the liquor free from waste material. Furthermore it must be noted that all these abovementioned substances have, in other parts of the body, important supportive

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properties and the presence of one or several of them is probably necessary in order to maintain the fibre form.

Secretion in fibre form. Most of the reactions which have been proposed here involve the formation of different kinds of end-products ("old" polysaccharide molecules, inactivated toxins, etc.), which must be removed from the cerebrospinal fluid. This fact may explain the curious and unusual form and consistency of the subcommissural secretion. If the reactions take place in this rod of semi-liquid secretion, instead of freely in the fluid, the end-products are gradually being transported caudally, removed from the central nervous system at its extreme caudal end, and finally broken down.

Animals without fibre apparatus. It may seem contradictory to assert the importance of a fibre apparatus for the liquor reactions, when some of the most advanced vertebrate brains totally lack this structure. It must be remembered, however, that the proportion between the cerebrospinal fluid and the nervous tissue volumes is much smaller in advanced brains than in primitive ones, which may mean that the importance of the cerebrospinal fluid for the brain metabolism decreases in the higher vertebrates (cf. Oksche 1957). To this must be added the fact that the renewal of the fluid is a slow process in the primitive brain, while the fluid of the mammalian brain is renewed several times a day. It seems reasonable to believe that a structure with the function of purifying the liquor must have lost much of its importance for the brain of the higher vertebrates and that, for this reason, the Reissner's fibre apparatus has gradually become less important during the phylogenetical development of the vertebrate brain, until finally, in some cases among the mammalians, it has become superfluous.

THE FUNCTION OF THE HYPENDYMAL ROSETTES

It is possible that the subcommissural ependymal cells release secretory material also into the blood vessels. But be this as it may, in so far as the subcommissural hypendymal cells are concerned, however, such a basal release seems much more probable. The blood vessels which are in contact with these cells apparently lack perivascular spaces, which means that a basal release of these cells is a release into the blood stream.

We must look upon the rosette cells as a specialization of fibre-forming cells, but not as their substitute. The rosette granules give the same histochemical reactions as Reissner's fibre, but this fact does not allow us to draw any other conclusions than the existence of some similarities in the composition of both kinds of secretion. Apparently the lower vertebrates have only fibre secretion while some mammals have both kinds of secretion (*Canis, Bos*) and finally there are signs that the rosettes may survive the fibre apparatus (*Homo*).

The subcommissural rosettes are as yet too badly known to allow any guessing about their function. Today it must suffice to establish the fact that ependymal rosettes (whatever their origin) *may* play an important role as glands. According to CASTIGLI (1941) rosettes develop in the pineal gland of *Lepus*, but are later on replaced by glial cells, with the result that the secretory activity of the pineal gland ceases.

FINAL REMARKS

The phylogeny of the subcommissural organ, as proposed in this paper, affords a striking parallel to that of the thyroid. Both have once been organs (*infundibular, flexural organ—endostyle, hypobranchial groove*) producing a mucous secretion into a cavity (*brain cavity—pharynx*). This substance has a tendency to form fibres, which transport material (*liquor waste—food particles*) to a caudal place where it is broken down. The transport mechanism has become superfluous during the evolution, owing to certain changes affecting the original cavity (*better liquor flowing—abandonment of ciliary feeding*), but part of the old exocrine gland has been preserved, withdrawn from the original cavity and transformed into an endocrine gland (*subcommissural rosettes—thyroidea*). This radical change in function has, however, taken place much earlier in the case of the thyroidea (at the cyclostome level), while as regards the subcommissural organ, it just seems to be in progress among the mammalians.

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SUMMARY

Although a detailed fibre analysis was carried out, it was not possible to establish a special innervation of the subcommissural organ in *Polypterus*. The secretory material released by this organ is very rich in cysteine and probably contains sialic acid too. In primitive vertebrates this secretion is apparently delivered into the brain cavities only, where the ventricular fluid movements condense it to Reissner's fibre. The appearance of the fibres in cases of duplicitas anterior (*Salmo*) makes it probable that their consistency is that of a viscid fluid, which slowly flows caudally. In living specimens (*Leuciscus*) it was possible to observe this substance leaving the central nervous system at its caudal end. It is proposed that the fibre acts as a detoxicator of the cerebrospinal fluid and it is also possible that the fibre substance may take an active part in enzyme activities. In advanced vertebrates also a basal

secretory release of the subcommissural cells is possible, that is, a release to blood vessels. In some mammals (*Bos*) several subcommissural cells have withdrawn from the ventricular position and form rosettes in the hypendyma. They still produce a secretory material with the same staining properties as that of the fibre-forming cells but now apparently exclusively to the blood vessels. Ependymal rosettes of subcommissural origin are found not only in the hypendyma but also in the caudoventral part of the pineal organ. It is likely that part of the subcommissural organ may be preserved in the form of pineal ependymal rosettes also in species which as adults totally lack the fibre-forming apparatus, as man.

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STUDIES ON THE FORMATION OF THE FERTILIZATION MEMBRANE IN SEA URCHINS

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The formation and properties of the fertilization membrane have been subject to extensive studies. The reason for this interest is the fact that the fertilization membrane "constitutes an easily visible indication of the underlying surface changes initiated by the fertilization reaction" (10).

Already from early studies it is well known that the fertilization membrane of sea urchin eggs is fragile during its elevation (10). The toughening which occurs within some minutes was more systematically studied by MOTOMURA (17). He centrifuged or shook the eggs after different treatments and estimated the fraction of eggs in which the membranes had been broken up. With a cell elastimeter MITCHISON an SWANN (13 a) have measured the stiffness of the egg surface at fertilization, but the study concerns little with the fertilization membrane.

In this paper a method for continuous "quantitative" analysis of the "membrane toughening" is described. The technique is based on mechanical removal of the fertilization membrane by filtering through bolting silk (11, 6). Some applications are made which may throw additional light on the fertilization membrane and its formation and thus upon problems of fertilization and development.

MATERIAL AND METHODS

In the studies carried out at Kristinebergs Zoologiska Station, Fiskebäckskil, the littoral Z-form of *Psammechinus miliaris* was used. The material used in Stazione Zoologica, Naples, was *Paracentrotus lividus* and *Psammechinus microtuberculatus*.

The animals were opened by a circumoral cut and the gonads washed in situ with filtered sea water. The ovaries shed eggs within a few minutes after being transferred into a small amount of filtered sea water. The eggs were separated from the ovaries by filtering through coarse bolting silk and washed by sedi-

mentation in filtered sea water. Their quality were tested by observations of fertilization in the phase contrast microscope. When jelly-free eggs were needed the jelly coats were removed mechanically (6).

Dry sperm shed from the removed testes was collected by pipetting. It was stored in small test tubes at about 10° C up to 6 hours. Within 10 minutes before insemination the dry sperm was diluted 200 times with filtered sea water. The sperm solution was further diluted in a beaker, agitated, and insemination performed by rapidly pouring into it about 5 times the volume of agitated egg suspension. The final dilution of dry sperm was $5.0 \cdot 10^{-5}$, and the final concentration of eggs was about $1 \cdot 10^{6}$ per litre. The experiments were performed in a temperature of about 16°C.

For quantitative analysis of the membrane removal, the LINDAHL—LUNDIN apparatus was further modified. The suction device (Fig. 1 A) was thus substituted by a pressure apparatus (Fig. 1 B). The 2 cm wide glass tube (GT)—its lower end covered with bolting silk—is the same in both methods devised. In the pressure apparatus its upper end is connected with the content of a stock vessel (SV) by a siphon (Si) closed by a jam. The glass tube and the siphon are filled with sea water and fixed to a stand, while the stock vessel hangs freely by a spring (Sp) so adjusted as to keep the difference between the water level and the bolting silk constant when the stock vessel is emptied.

The experiment routine was to pour 100 ml egg suspension in about 300 ml filtered sea water in the stock vessel 60 seconds after insemination. The eggs were gently stirred by a motordriven (Mo) glass rod. Sampling was achieved at fixed intervals—usually every 20th second—by the jam into a series of small beakers (SB). These contained some sea water, and during the sampling—which lasted about 3 seconds—the bolting silk was kept just under the surface of the sea water.

Bolting silk[1] of the same mesh dimensions as used for the removal of the jelly coats was used for the removal of the fertilization membranes. Thus the meshes are just a little smaller than the diameters of the eggs: the mesh number used for *Paracentrotus* was 16 and for *Psammechinus* 14. For removal of the jelly coats the suction was adjusted to give a filtering rate of about 15 ml egg suspension per second per cm^2 bolting silk. For quantitative analysis of fertilization membrane removal the pressure had to be kept within a rather limited range in order to obtain high sensitivity (Fig. 3): for *Paracentrotus* the filtering rate was about 7 and for *Psammechinus* about 5 ml per second per cm^2 bolting silk.

In each sample about 200 eggs were counted before the second cleavage under phase contrast microscope. In later stages damaged membranes shrink

[1] from Schweiz. Seidengazefabrik, Thal/St. Gallen.

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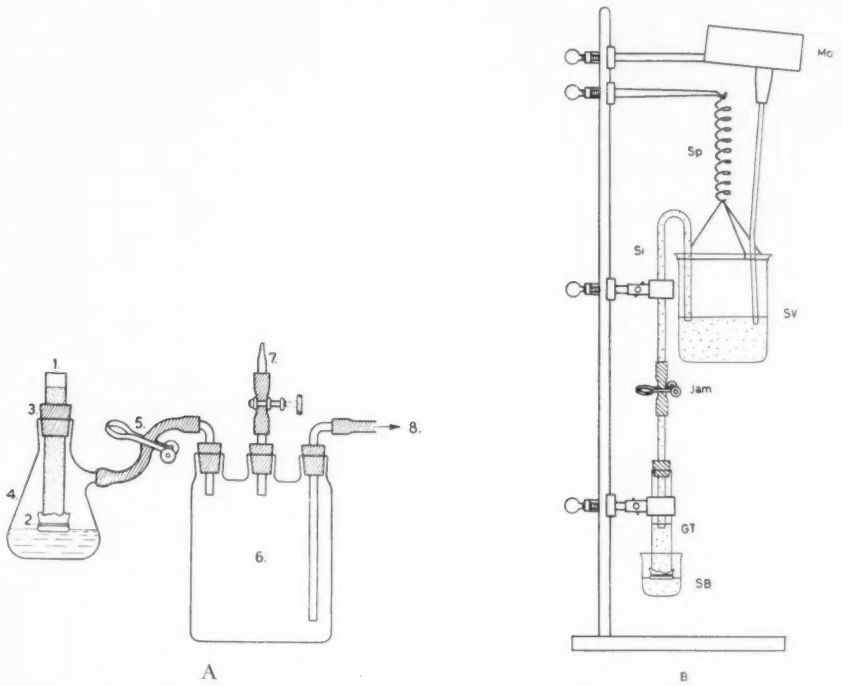

Fig. 1 A. Suction device for removal of fertilization membrane or jelly coat. From (6).

Fig. 1 B. The pressure device for fertilization membrane test. GT Glass Tube with the bolting silk, Mo Motor for agitation, SB Sampling Beaker, Si Siphon, Sp Spring to keep the pressure constant, SV Stock Vessel.

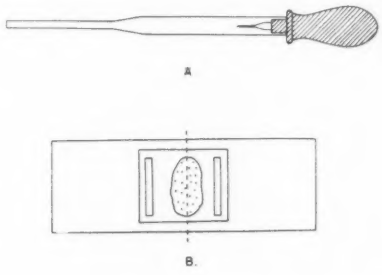

Fig. 2. A. Pipette with inserted capillary tube to secure a slow suction.

Fig. 2. B. "Counting chamber". The countings were made along the dotted line.

and thus counting becomes more difficult. The conditions for taking the eggs from the sampling beakers and delivering them to the "counting chambers" were analysed to assure high accuracy. Reproducible results (Table 1 A) were obtained by sampling across the diameters of the quietly sedimented egg layer in the sample beaker with a slow suctioning pipette—a capillary tube is inserted in the upper end of the pipette (Fig. 2 A). The sample was then

delivered quantitatively as an elongated drop on to an object slide. A cover slip was placed over the drop on small edgings (Fig. 2 B) and the counting was done along the central axis of the drop (dotted line in the figure).

The percentage of eggs with their membranes still intact were determined and plotted against time in diagrammes. Also eggs with damaged membranes were included. By repeating one experiment three times the accuracy of this fertilization membrane test was checked (Table 1 B).

TABLE 1.

A. Accuracy of the counting procedure. Three repeats of sampling have been made from each sampling beaker in a control experiment.

B. Accuracy of the whole fertilization membrane test. A control experiment have been repeated three times.

A

Time after insem.	% eggs with intact membranes and Number of eggs counted.					
Min. Sec.	%	N	%	N	%	N
1 40	68	247	72	166	74	147
2 00	66	291	67	154	67	245
2 20	63	358	60	179	64	165
2 40	55	152	52	151	51	155
3 00	38	224	38	139	35	201
3 20	49	183	54	212	46	213
3 40	68	215	72	162	71	154
4 00	81	203	83	194	79	146
4 20	89	166	91	142	93	140
4 40	92	282	94	128	91	196

Variation	Degress of freedom	Mean square deviat.	Standard deviation
Within the experiments	9	957	
Between the experiments	20	5	± 2,5 %

B

Time after insem.	% eggs with intact membranes and Number of eggs counted.					
Min. Sec.	%	N	%	N	%	N
1 40	67	119	72	137	74	185
2 00	62	162	70	103	65	152
2 20	44	177	42	100	40	134
2 40	29	238	25	97	26	149
3 00	29	150	30	103	24	124
3 20	61	127	68	96	59	160
3 40	87	107	89	115	81	125
4 00	97	125	100	—	95	120

Variation	Degress of freedom	Mean square deviat.	Standard deviation
Within the experiments	7	2038	
Between the experiments	16	12	± 3,5 %

In some experiments the diameters of the elevated membranes were determined. It was done soon after its final hardening by measuring two diameters at right angles of each egg in about 15 eggs of every batch.

Great care was taken to make sure that the control tests were handled in the same way as the experimental tests and only differed in the treatment in question. All glasswares were of the same quality and were washed in the same way. Detergents were never used for washing, and besides water only concentrated sulphuric acid was used when necessary.

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RESULTS

The membrane test curves present rather irregular features during the first two minutes after insemination. Therefore emphasis will be laid on the part of the curves for times later than two minutes after insemination. The usual type of the curve for the first two minutes will however be illustrated (Fig. 4).

As is seen from control curves (e.g. Fig. 3) about 60 % of the eggs filtered 2 minutes after insemination have membranes—though many of them are damaged—while the rest of the eggs are quite naked. A sharp minimum is found about 3 minutes after insemination. Here normally only about 20 % of the filtered eggs have membranes. From this point the curve steeply rises to values where over 90 % of the eggs have intact membranes after filtration. From 5 minutes after insemination no essential changes in the rigidity of the fertilization membrane were noticed until time of hatching. The empty membranes were found in a considerable number among the sedimented eggs. Their size was apparently of the same order as the egg surface, and they were more or less wrinkled and had usually a linear rupture of about two thirds of the diameter.

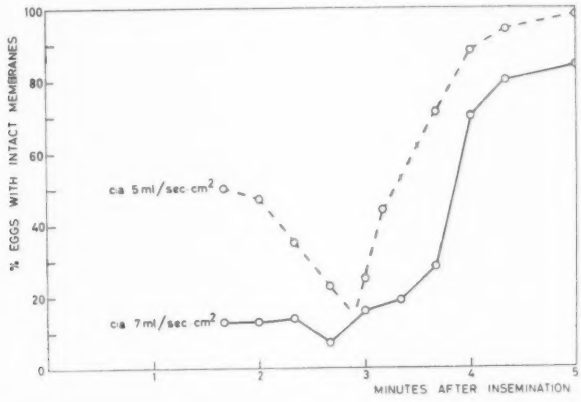

Fig. 3. Influence of different filtering rates on membrane removal on Psammechinus microtuberculatus.

As follows from experiments with different filtering rates the fertilization membranes are more fragile in eggs of *Psammechinus miliaris* and *microtuberculatus* than in eggs of *Paracentrotus*. In other respects the curves are similar. An increased filtering rate depressed most of the curve obtained under optimal conditions and levelled the differences with the exception of the ascending part of the curve (Fig. 3). These experiments indicate that if the units of the

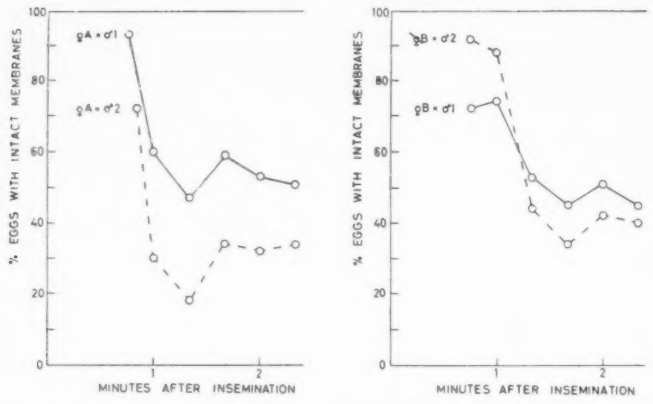

Fig. 4. Fertilization membrane test on Paracentrotus lividus for the first minutes after insemination. Two females tested against two males. The probability for only random differences between the two females is 0,01 and <0,001 for respective male, and between the two males it is <0,001 and 0,01 for respective female.

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ordinate are standarized the ascending part of the curve is more than three times higher than the descending part.

In a few experiments with *Psammechinus* eggs spermatozoa were observed within the perivitellin space. Such observations were rarely done in experiments with *Paracentrotus* eggs. This may constitute another indication of the higher fragility of the *Psammechinus* membranes.

In some experiments 5 to 10 per cent of eggs deprived of their fertilization membrane were polyspermic, whereas less than 1 per cent of eggs with intact membranes showed polyspermy. This was however the case only for the earliest samples in these experiments, and later than two and a half minutes after insemination no increase in polyspermy was observed.

Even under normal conditions the membrane test curves present considerable variations in the interval 2—5 minutes after insemination. In order to study this natural variation, different males were tested against one female and the reverse different females against one male. As may be concluded from the figures 4 and 5 this variation of the curves does not only reflect properties of the eggs but also those of the sperm.

There is apparently a connection between an atypical form of membrane test curve and later abnormal trends in the development. Clear evidence of this was given in certain experiments where eggs used for membrane test were cut in animal and vegetal halves during the 16 cell stage. Later some or all of the operated larvae cytolysed in experiments with eggs where the fertilization membrane test curves indicated a membrane formation deviating from the normal (Fig. 5 and Table 2).

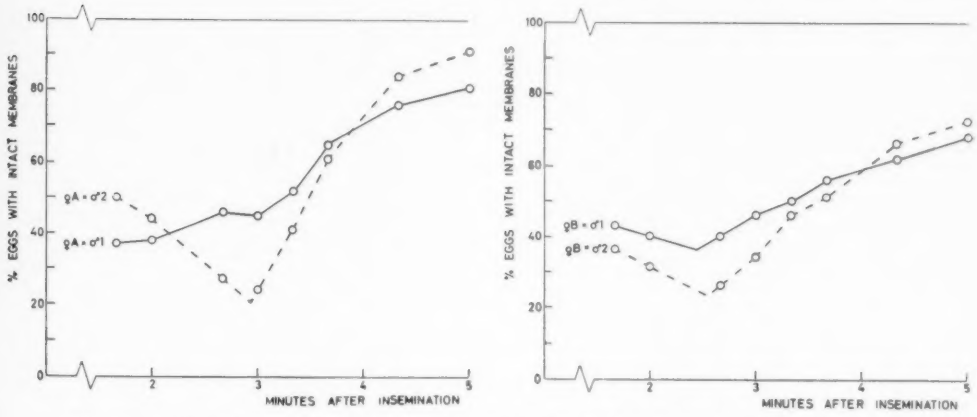

Fig. 5. Fertilization membrane test on Paracentrotus lividus. Two females tested against two males. The probability for only random differences between the two females is for respective male 0,1 and 0,01, and between the two males the corresponding figures are 0,001 and 0,05.

TABLE 2.

Correlation between membrane test curves in Fig. 5 and results of operation.

	Number of operated larvae.	12 hours after insemination.	31 hours after insemination.
♀A × ♂1	18	3 alive	Total cytolysis
♀A × ♂2	15	all alive	all alive
♀B × ♂1	24	Total cytolysis	—
♀B × ♂2	13	all alive	10 alive

The influence of changes in the chemical environment was tested in some preliminary experiments. All tests here mentioned were made with jelly free eggs, and it was ascertained that membrane elevation had started in almost all eggs at the time for changing the chemical environment.

A decrease in calcium content of sea water to about 1/5 was achieved by pouring one volume egg suspension into four volumes of calcium free artifical sea water of the same salinity. This was made one minute after insemination in the stock vessel of the filtering apparatus. Lack of calcium ions (Fig. 6) caused an increased loss of membranes in an early phase. Later the sharp minimum point of the curve was prevented by a decrease in membrane removal. Finally the ascending part of the curve was strongly inhibited or delayed. In these experiments lack of calcium increased the membrane diameters.

In other experiments the eggs were subjected to trypsin. The treatment was set in one minute after insemination by pouring the egg suspension in the stock vessel solution containing a crystalline preparate of trypsin to a final concentration of 1—10 mg per 100 ml suspension. The first part of the curve was

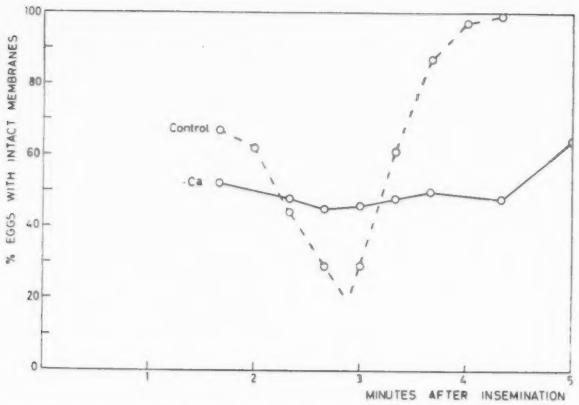

Fig. 6. Influence of lack of calcium ions on the fertilization membrane test of Psammechinus miliaris. The probability for only random differences between the descending parts of the curves is 0,005 and for the ascending parts <0,001.

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flattened in a way similar to that resulting from a lack of calcium (Fig. 7 A), whereas in any case the weaker solutions seemed not to affect the ascending part of the curve. There was a slight decrease in membrane diameters after treatment with trypsin in these experiments.

When the eggs were subjected to an about $2 \cdot 10^{-6}$ molar solution of copper (II) ions one minute after insemination the ascending part of the curve was

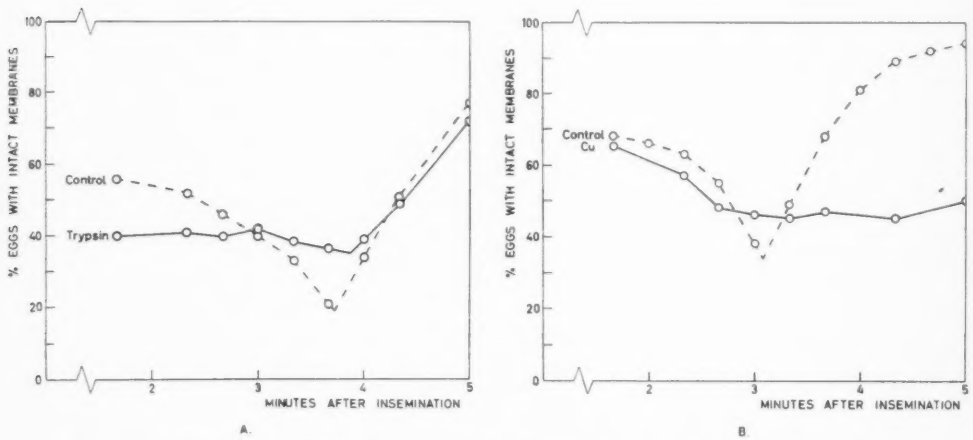

Fig. 7 A. Influence of trypsin on the fertilization membrane test of Paracentrotus lividus. The probability for only random difference between the descending parts of the curves is 0,01, and between the ascending parts there is no statistical difference.

Fig. 7 B. Influence of copper (II) ions on the fertilization membrane test of Paracentrotus lividus. The probability for only random differences between the descending parts of the curves is 0,2 (little statistical difference) and for the ascending parts <0,001.

clearly flattened (Fig. 7 B). In this study copper seemed to decrease the membrane diameters to some extent.

Pretreatment of the unfertilized eggs in a $5 \cdot 10^{-6}$ molar solution of potassium periodate for 10 minutes was also studied. In some cases there was an increased percentage of eggs with intact membranes throughout the whole period observed. In other experiments the difference was slight if any, and in a few experiments there was a tendency to increased fragility.

DISCUSSION

The early membrane has been shown to have dialysing and elastic properties (15, 8). Evidence was given (12, 8) for the active mechanism of the elevation to be the development of a colloid osmotic pressure within the perivitellin space. When the elastic force of the membrane balances the osmotic pressure elevation stops (8).

The wrinkling of the egg surface in the beginning of membrane elevation seems to be a consequence of the developing osmotic pressure in the perivitelline space. This wrinkling is an occasional and temporary phenomenon often connected with under-ripeness and deviations in membrane formation (21). HIRAMOTO (8) suggests that the concavity of the egg surface at the point of sperm entrance also is due to the development of a pressure inside the membrane.

The perivitelline space contains elastic structures as has been intimated by HIRAMOTO (7). He also indicated that the presence of calcium is necessary for the development and maintenance of these structures which keep the egg in a central position within the perivitelline space. In some batches of eggs from *Psammechinus microtuberculatus* radiant strands in the perivitelline space were observed by means of phase contrast microscopy. The same phenomenon also occured in *Echinus esculentus* from the Swedish westcoast and seemed to be a rather normal property in eggs of *Genocidaris maculata* from the gulf of Naples. These strands may be an evidence of the elastic structures.

Shortly after membrane elevation the membrane hardens (10, 17). In absence of calcium ions this toughening fails to occur (17, 9). Another substance necessary for membrane toughening has been extracted from the eggs by acid treatment (17). It may be worth mentioning that the acid released upon fertilization (2) may coincide with membrane toughening.

Enzymatic steps among the events in connection with fertilization and membrane formation was early suggested (cf. 21). LUNDBLAD (13) has given a survey of proteolytic enzymes which are activated on fertilization and operative within the first ten minutes. This seem to throw light upon the effect of calcium and upon pretreatment with periodate (23, 24). The varying results with periodate obtained in this paper may be due to the state of ripeness of the eggs

which is of great importance (24), but also to the instability of periodate ions in sea water (cf. 23) which thereby induce difficulties in obtaining reproducible treatment conditions.

When the eggs are forced by the water pressure through the narrow meshes of the bolting silk, there is a tendency of the fertilization membrane to rupture. This must be due to local tensions in the membrane caused by friction between the membrane and the bolting silk. The "friction" may also be of electrical nature and reside on charges in the membrane and in the bolting silk. There is however evidence for the view that the mechanical friction alone is important in this respect. No removal of the fertilization membrane is obtained if nylon is used instead of silk as material in the gauze. The meshes are of the same size and form, the nylon threads are however of a very smooth appearence. Moreover, there is never any accumulation of empty membranes to the bolting silk which would have been expected if electrical forces were responsible for the rupture of the membranes.

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It may be assumed that the percentage of removed and damaged membranes in this investigation is a measure of the tendency of the fertilization membrane to rupture. It was shown above, that changes in the electrical charges of the fertilization membrane don't seem to be responsible for the results, and other interpretations will here be considered more likely than changes in the friction coefficient of the membrane. On the other hand the form of the curve not necessarily depends on changes in the physical and chemical properties of the membrane.

If the earliest part of the curve should be considered, the first decrease of the curve (Fig. 4) shows the beginning of membrane elevation. The following increase in rigidity may indicate some building up of the early membrane, e.g. an incorporation of cortial material (20, 3).

The descending part of the curve (e.g. Fig. 6) starts after full elevation of most membranes. Besides their size, tension in the membranes caused by the generating osmotic pressure may contribute to this part of the curve. The development of the perivitelline structure should also be of great importance for increasing the mechanical distortion in the membrane of an egg passing through the bolting silk. Evidence for this seems to be the flattening of this part of the curve in a calcium free medium.

The ascending part of the curve seems open to a simple interpretation. A rapid process of toughening of the fertilization membrane starts at about the minimum point. It renders the membrane more and more resistant to the mechanical distortion during the passage through the meshes. The process is finished about five minutes after fertilization. Previously the necessity of calcium for membrane toughening has been mentioned, and the effect of copper on membrane removal may also indicate enzymatic steps.

The minimum of the membrane test curve seems to be obtained by the intersection of the descending and the ascending lines. This occurs between two and three minutes after fertilization.

The results from different males tested against different females indicate the curve of the membrane test to be a product of both egg and sperm factors. It means that the fertilization reaction quantitatively or qualitatively is affected by the sperm. That the state of the sperm influences the elevation of the membrane has several times been suggested (21, 25, 26) but the underlying methods have not been quite conclusive. Recently, however, increase in membrane elevation by fertilization with heterologous sperm was demonstrated (5).

Of importance for the understanding of the sperm influence on membrane formation seems to be the suggestion that the cortical reaction is not an all or non reaction. This is in line with the view discussed by ALLEN and HAGSTRÖM (1) who also indicated that chemical factors may influence the cortical reaction beside physical factors. It seems here tempting to suggest that substances released from the sperm migrate in the egg surface, a suggestion which should be compared with the view of ROTHSCHILD and SWANN (19). In agreement with the correlation between membrane test curves and subsequent development—as reflected by larval resistance to operation—is the view that endoplasmic reorganization is mediated indirectly through activation of the cortex (cf. 1).

The rareness of sperm cells within the perivitelline space might indicate that the fertilization membrane participates in the block to polyspermy. Other investigations (cf. 4 and 18) have shown that other mechanisms are of dominating importance—and the occurence of sperm cells within the fertilization membrane without increased polyspermy also indicates other blocks. Not much attention should be laid on the occasional increase of polyspermy in filtering experiments, because thereby the eggs have been subjected to a mechanical treatment in a period when they are known to be very sensitive.

SUMMARY

1. A method was developed for continuous estimation of the "mechanical resistance" of the fertilization membrane of sea urchin eggs (*Psammechinus miliaris* and *microtuberculatus* and *Paracentrotus lividus*). At different intervals after insemination the eggs were filtered under light pressure through bolting silk. The percentage of filtered eggs with their membranes still present was then estimated. The accuracy of the method was proved to be satisfactory.

2. The time curve of the membrane resistance has a pronounced minimum

about 3 minutes after insemination. After this minimum the resistance increases rapidly and reaches its final strength about 5 minutes after insemination.

3. The natural variation of the curves is studied and attention is called to the influence of sperm factors as well as to the correlation to subsequent larval development.

4. In sea water deficient in calcium ions the curves lack the minimum point and the resistance remains on an almost constant level. Preliminary data are also given concerning the effect of trypsin, of copper and of periodate.

5. Attempt is made at an intepretation of the form of the curve.

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THE DEVELOPMENT OF THE CHONDROCRANIUM OF PYROMELANA ORIX ORIX

BY

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(Zoological Institute, University of Stellenbosch)

(With 32 text-figures)

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PART I

I INTRODUCTION

This paper is one of a series in which the results of investigations on the cranial morphology and ontogeny of birds, carried out in the Zoological Institute of the University of Stellenbosch are described. The earlier papers are those by SWART (1946), PRINS (1951), CROMPTON (1953), FRANK (1954), DE KOCK (1955) and FOURIE (1955). A paper by Webb is at present in the press while another by DE KOCK is in preparation.

The avian chondrocranium received considerable attention from W. K. PARKER who was one of the outstanding workers on the development of the vertebrate skull during the second half of the last century. Although microscopical technique was not nearly as advanced as today, very few structures in the avian chondrocranium escaped the attention of this experienced anatomist. Unfortunately Parker worked at a time when the nomenclature was not yet standardized so that his descriptions are often difficult to follow.

An excellent paper by SUSCHKIN on the development of the chondrocranium and dermal bones of *Tinnunculus* was published in 1899. Suschkin's investigation covers practically the entire development. Apart from the detailed description of the position and histogenesis of every part of the chondrocranium, Suschkin's work is also outstanding in the detailed review of previous literature. He was followed by SONIES (1907) whose researches on the development of the chondrocranium of the duck and fowl brought to light a great deal of new information. With improved technique he was the first to observe separate anlagen for the cochlear and canalicular portions of the otic capsule and the presence of polar cartilages in birds. A more detailed account of the development of the duck was given by DE BEER and BARRINGTON (1934). Their main contri-

bution lies in the standardization of the nomenclature and the discussion of the morphological problems presented by the avian chondrocranium.

Recent contributions are the papers of CROMPTON (1953) and FRANK (1954) on the craniogenesis of the penguin and the ostrich respectively. Crompton paid special attention to the development of the columella auris. Except for the description of a few developmental stages of the ostrich by BROCK (1937), Frank's paper is the first detailed account of the development of the chondrocranium of a dromoeagnathic bird since T. J. PARKER'S (1891) paper on the development of *Apteryx*.

Our knowledge of the development of the avian chondrocranium is still inadequate, and before the development of at least one example of order has been studied it is dangerous to regard one form as being more primitive or specialised than another. Apart from the lack of knowledge the variable mode of formation of structures characteristic of the avian chondrocranium such at the metotic cartilage and the infracarotid commissure makes it difficult to establish affinities.

The first part of this paper is a description of a number of developmental stages, covering the development of the chondrocranium prior to ossification. Special attention has been paid to the development of the ethmoidal region and to the process of resorption in this region. The second part is a summary and a discussion of certain morphological problems involved.

II MATERIAL AND TECHNIQUE

The material for this investigation was collected along the lower part of the Orange River where the Red Bishop Bird *(Pyromelana orix orix)* occurs in large numbers. In the vernacular this bird is known as "Red Finch" of "Kaffir Finch". These names are incorrect as it is not a true finch but a weaver (Fam. *Ploceidae*).

Formalin, Allen's Fluid and sublimate were employed as fixatives. Excellent fixation was obtained with all these but staining results were better in material fixed in sublimate. The specimens were classified according to length (tip of beak to tip of tail). For decalcification a solution of concentrated nitric acid in 70 % alcohol in the proportion 6 : 100 was employed. Serial sections were made of a large number of specimens ranging from young embryos to old nestlings as well as of an adult. The older nestlings were sectioned transversely, while for the younger stages enough material was available for both transverse and sagittal sections of each stage. Sections were cut at 10μ, 15μ and 20μ depending on the age of the specimen.

Various methods of staining were employed. As the intercellular substance of the cartilage of the embryos showed practically no affinity for azan, embryos

were stained in toto with Mayer's basic haemalum and the sections counterstained with Bismarck brown and eosin. Unfortunately nerves are very poorly stained by this combination. A variation of this method, in which eosin was omitted, proved to be very useful for detecting the first traces of intercellular deposit. Nestlings were all stained in toto with borax carmine and the sections counterstained in azan.

Reconstructions were made according to PUSEY's (1939) methods. Wax-plate models of the complete chondrocranium of stage IV, of the nasal capsules of stages VI and X and of the basal plate and otic capsules of stage VIII greatly facilitated the work.

For comparison I had at my disposal the Ziegler copy of W. TONKOFF's modell of the chondroranium of an embryo of *Gallus*, models of various stages of the chondrocranium of embryos of *Spheniscus demersus* made by A. W. CROMPTON, and of the nasal capsules of *Struthio* and of the South African nightjar *(Nyctisyrigmus pectoralis pectoralis)* made by G. H. FRANK.

Series of developmental stages of *Anas* sectioned by F. J. GREWE, *Spheniscus demersus* sectioned by A. W. CROMPTON, and *Struthio* sectioned by G. H. FRANK as well as an embryo and a nestling of *Nyctisyrigmus* sectioned by Dr. M. E. MALAN of this Institute were available for study.

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III ACKNOWLEDGEMENTS

This investigation was carried out under the supervision of Prof. C. G. S. DE VILLIERS whom I wish to thank most sincerely for his personal interest and advice during the time I have been in this Institute. I am also very much indebted to Dr. M. E. MALAN for her advice and valuable criticism in the preparation of this paper.

IV DESCRIPTION OF DEVELOPMENTAL STAGES

A STAGE I

Total length of embryo: 18 mm.

1 *The Basal Plate and Otic Capsules*

The basal plate and acrochordal cartilage have the typical S-shaped form of young avian chondrocrania, a condition first described in *Tinnunculus* by SUSCHKIN (1899). The basal plate and lower portion of the acrochordal are traversed by the notochord. In the acrochordal and anterior part of the basal plate the notochord is situated nearer to the cerebral surface of these structures.

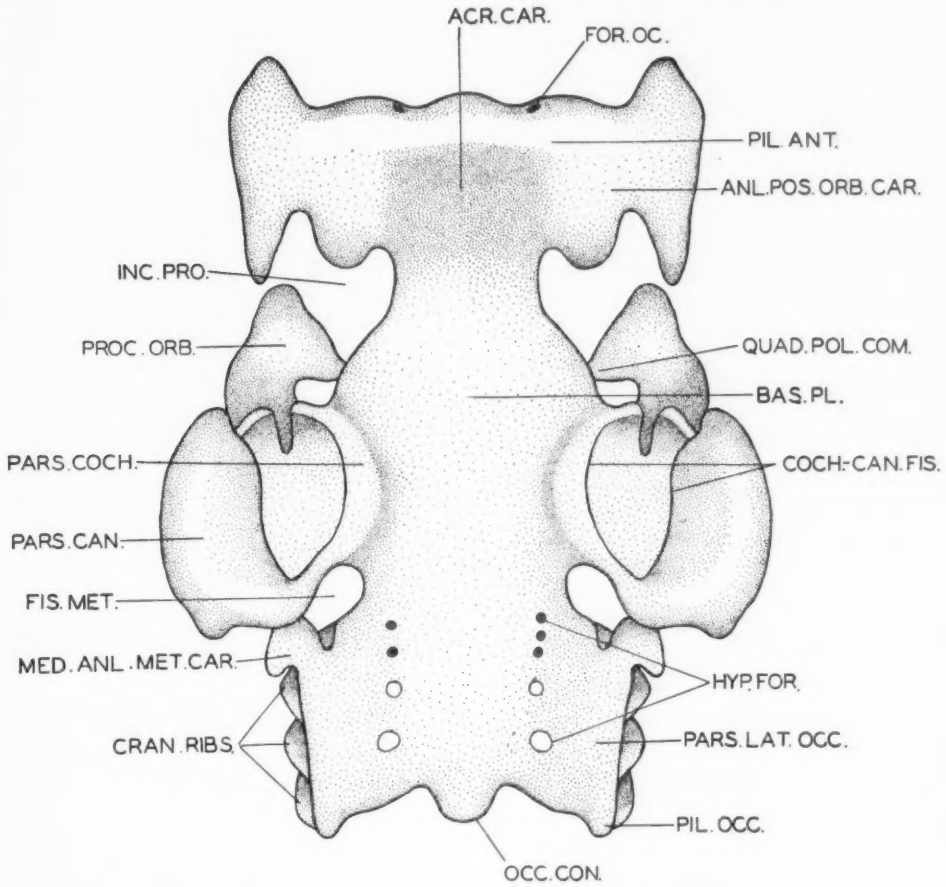

Fig. 1 (× = 48.6). Stage 1. Reconstruction of chondrocranium (norma dorsalis).

For a short distance behind this it occupies a central position in the basal plate, while farther posteriorly it is situated nearer to the cerebral surface. The anterior tip of the chorda is flexed downwards and unlike conditions in *Tinnunculus* (Suschkin, 1899), *Anas* and *Gallus* (Sonies, 1907), *Dromiceius* (Lutz, 1942), *Spheniscus* (Crompton, 1953) and *Struthio* (Frank, 1954) it does not pierce the anterior surface of the acrochordal, although it does so very nearly. The chorda is of more or less equal diameter throughout its length and shows a few vertical undulations in the anterior tip and in the anterior region of the basal plate.

Fused to the basal plate laterally are the anlagen of the otic capsules (Figs. 1 and 2). In each anlage a cochlear and canalicular part can be easily distinguished (Fig. 1). The former is procartilaginous and completely fused to he cartilagi-

nous basal plate. The pars canalicularis is histologically less differentiaed and consists of blastematous tissue containing intercellular deposit. Both anlagen are cup-shaped, their concavities facing each other. A floor and posterior wall connect the two entities (Fig. 1), the posterior wall corresponding to the dorsal cochleo-canalicular commissure described in the penguin by CROMPTON (1953). A large vacuity lies between the edges of the roofs of the canalicular and cochlear portions (Fig. 1). Through this vacuity, which represents the dorsal part of the cochleo-canalicular fissure, the ductus endolymphaticus leaves the capsule, and all the branches of the acustic nerve enter the capsule.

Anteriorly a deep fissure is present between the canalicular and cochlear portions (Fig. 1). It is bordered posteriorly by the floor connecting the two parts, medially by the lateral edge of the front-wall of the pars cochlearis and laterally by the medial edge of the front wall of the pars canalicularis. Through this fissure which represents the antero-ventral part of the conchleo-canalicular fissure the facial nerve passes forwards. A similar course for the facial nerve has been described in the ostrich (BROCK, 1937 and FRANK, 1954) and penguin (CROMPTON, 1953). Anteriorly this fissure is continuous with the large incisura prootica (Fig. 1).

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When seen from below the basal plate is pierced on each side by three hypoglossal foramina, the diameter of the foramina decreasing from back to front. In the 2nd and 3rd hypoglossal roots the twigs unite before leaving the cranial cavity through their corresponding foramina. In the first hypoglossal root the twigs pierce the dorsal surface of the basal plate separately and join inside the plate.

On the one side two, and on the other side three foramina for the twigs of the first hypoglossal roots are present on the cerebral surface of the basal plate (Fig. 1). On its ventral surface each anterior root emerges as one nerve through a single foramen.

Lateral to the foramina, the occipital arches lying between them are fused into a cartilaginous plate, the pars lateralis of the occipital region, overlying the occipital myomeres (Figs. 1 and 2). Strictly speaking the hypoglossal foramina are thus situated between the pars basalis of the occipital region of the basal plate and the pars lateralis which are formed by the fused distal ends of the occipital arches lying between the hypoglossal roots. From the antero-lateral corner of the pars lateralis of the occipital region a procartilaginous plate projects antero-laterally beneath the floor of the pars canalicularis of the otic capsule (Figs. 1 and 2). This is the medial anlage of the metotic cartilage. The lateral anlage of the metotic cartilage has not yet been differentited as a definite structure and is included in the thickened lateral part of the floor of the pars canalicularis of the otic capsule. The glossopharyngeal and vagus-accessory nerves leave the cranial cavity through the metotic fissure (Fig. 1) bordered medially by the lateral edge of the basal plate, posteriorly by the anterior edge of

the pars lateralis of the occipital region of the basal plate, postero-laterally by the medial ledge of the medial anlage of the metotic cartilage and antero-laterally by the posterior wall of the otic capsule.

Attached to the ventral surface of the pars lateralis of the occipital region are three cranial ribs (Figs. 1 and 2), each of them lying behind one of the occipital myomeres. They are in the form of transverse ridges projecting antero-ventrally from the ventral surface of the pars lateralis. Their lateral tips project slightly beyond the lateral edge of the pars lateralis (Fig. 1). Medially they are continued uninterruptedly into the mesenchymatous myocommata between the more medial portions of the occipital myomeres. They are thus nothing more than the lateral portions of these myocommata in which some intercellular substance has been deposited. Histologically the cranial ribs differ from the myocommata not only in the possession of intercellular substance but also in the greater concentration of mesenchymatous cells in the latter.

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Three myomeres can be distinguished in the occipital region at this stage, decreasing in length and degree of differentiation from behind forwards. The small anterior myomere is attached anteriorly to the posterior surface of the medial anlage of the metotic cartilage.

Laterally on each side the cartilaginous acrochordal expands into a large structure consisting for the most part of a concentration of mesenchyme cells embedded in a very thin matrix of intercellular deposit. The medial portion of this structure is the pila antotica, the boundary between it and the acrochordal being indicated by the foramen for the oculomotor nerve (Fig. 1). The pila antotica is cartilaginous, but latero-dorsally it passes over into a diffuse structure without any definite boundaries, the anlage of the posterior part of the orbital cartilage (Figs. 1 and 2). It is impossible to ascertain the boundary between the pila antotica and the orbital cartilage.

In all birds investigated so far the pila antotica and posterior part of the orbital cartilage develop in continuity with the antero-lateral corner of the acrochordal, except in *Gallus* where SONIES (1907) claims separate anlagen for the pila antotica and posterior part of the orbital cartilage.

A wide incisura prootica (Figs. 1 and 2), bordered posteriorly by the otic capsule, medially by the lateral edge of the basal plate and anteriorly by the postero-ventral edges of the pila antotica and posterior part of the orbital cartilage, lodges the gasserian ganglion. Posteriorly above the floor of the otic capsule the incisura prootica is confluent with the cochleo-canalicular fissure (Fig. 1). Through the incisura prootica all branches of the tri-geminal nerve as well as the abducens leave the cranial cavity. The abducens is continued forwards close to the lateral edge of the basal plate and below the ventral edge of the pila antotica.

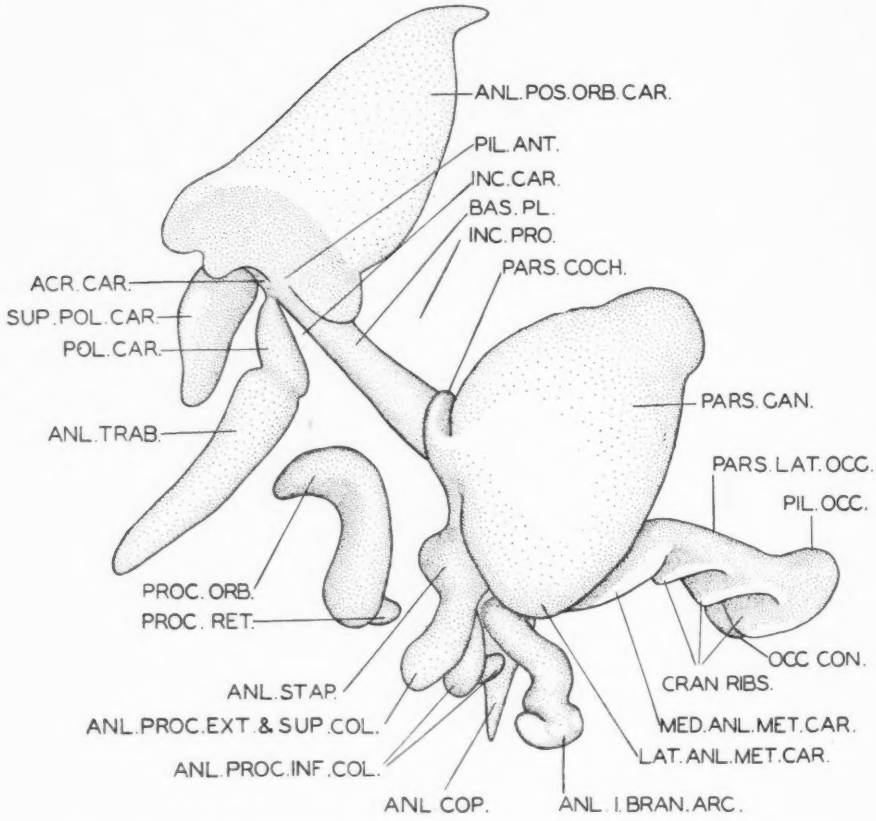

Fig. 2 ($\times = 48.6$). Stage 1. Reconstruction of chondrocranium (norma lateralis).

2 *The Anterior End of the Chondrocranium*

The only structure present in the prechordal region are the procartilaginous suprapolar elements and the trabeculo-polar bars. The latter are wide apart, their posterior ends fused to the anterior surface of the acrochordal and lateral to Rathke's pouch. Each trabeculo-polar bar consists of two easily distinguishable portions: the shorter procartilaginous posterior part is the polar cartilage while the anterior part of dense mesenchymatous tissue containing faint traces of intercellular deposit is the anlage of the trabecula. As can be seen from the reconstruction (Fig. 2), the polar cartilage and trabecular anlage are set at different angles to the longitudinal axis of the basal plate.

The angle between the basal plate and the longitudinal axis of the polar cartilage measures $\pm 45°$, while the angle between the basal plate and the longitudinal axis of the trabecular anlage is $\pm 97°$. Histologically the polar cartilage

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can easily be distinguished from the trabecular anlage: its cells are more rounded, and it is richer in intercellular substance; this is proved by its greater affinity for Bismarck brown. The trabecular anlage has no definite outline so that it is not possible at this stage to draw a very sharp line of demarcation between this anlage and the surrounding mesenchyme.

Posteriorly the polar cartilage is fused to the anterior surface of the acrochordal, but the line of fusion is still discernible. The hypophysial incisure, bordered posteriorly by the anterior surface of the acrochordal and laterally by trabeculo-polar bars, is wide open at this stage. Between the anterior surface and the ventral part of the acrochordal posteriorly and the ventral surface of the polar cartilage anteriorly is the incisura carotica (Fig. 2), traversed in a medial direction by the internal carotid artery. The latter passes in an anterodorsal direction medial to the polar cartilage to anastomose behind Rathke's pouch with its companion on the other side.

The suprapolar cartilage (SONIES, 1907) is a large elongated procartilaginous structure lying above the polar cartilage and posterior part of the trabecular anlage (Fig. 2). A deep incisure is present between the ventral edge of the suprapolar and the dorsal edges of the polar cartilages and posterior part of the trabecular anlage. Through the posterior part of this incisure the ophthalmic artery leaves the cranial cavity. DE BEER and BARRINGTON (1934) homologized the avian suprapolar element with the supratrabecular bar of lizards and primates. In *Lacerta* the supratrabecular bar develops late in ontogeny, while the suprapolar cartilages of birds appear very early. DE BEER and BARRINGTON (1934) thought that their early appearance in birds stood in relation to the attachment of the mm. recti oculi. In this stage the anlagen of the recti muscles are already attached to the procartilaginous suprapolars.

No trace of the intertrabecular element of the PARKER's and and SUSCHKIN (1899) is present in this stage.

3 *The Visceral Arches*

The cartilaginous sickle-shaped mandibular arch is situated immediately in front of the hyoidean visceral cleft and shows signs of differentiation into a dorsal pars quadrata and ventral cartilage of Meckel. Of the latter structure only the processus retro-articularis is present as a protuberance on the posterior surface of the ventral end of the mandibular arch (Fig. 2). Dorsally the pars quadrata bears an anteriorly directed processus orbitalis.

On the medial surface of the latter, a short distance behind its anterior tip, a dorso-medially directed procartilaginous process is present (Fig. 1). This anlage is the first indication of the quadratopolar connexion which will develop later.

The anlage of the columella auris is situated behind the hyoidean visceral

cleft which is still open. Its proximal part is procartilaginous and has a definite outline, but the more lateral part consists of dense mesenchyme only, without definite outlines and containing only faint traces of intercellular deposit. The mesenchyme cells are very densely packed in this region and there is no sharp boundary between the distal part of the columellar anlage and the surrounding mesenchyme. For this reason a reconstruction of this region of the columellar anlage does not pretend to be very accurate.

The columellar anlage can be divided into two parts, an outer mesenchymatous part from which the processus extracolumellaris and the processus supracolumellaris develop, and referred to in this paper as the extracolummellar part, and an inner procartilaginous stapedial part in which a stapes later ossifies. Histologically no sharp line of demarcation can be drawn between the two portions. The only indication of a future boundary is given by a medio-ventrally directed mesenchymatous processus infracolumellaris (Fig. 2). The medial tip of the stapedial part is fused to the floor of the procartilaginous cochlear part of the auditory capsule. Complete continuity of the intercellular substance exists between the two structures.

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Situated in an elevation in the midline of the floor of the oral cavity is the anlage of the copula in the form of a curved procartilaginous structure (Fig. 2). Externally it shows no sign of fragmentation but in sagittal sections, passing through and near the midline, a procartilaginous anterior and posterior portions divided by a thin zone of mesenchymatous tissue devoid of any intercellular deposit, can be observed. This is an indication of the future chondrification in the anlage of the first and second copulae. Projecting postero-laterally from this anlage and more or less on the boundary between the future copulae is the anlage of the first branchial arch (Fig. 2). Its proximal part is procartilaginous, and its medial tip passes over indistinguishably into the anlage of the copula. The distal portion is a mere rod of densely concentrated mesenchyme cells between which no intercellular substance has yet been deposited.

B STAGE II

Total length of embryo: 22.3 mm.

1 *The Basal Plate and Otic Capsules*

The basal plate has now broadened and is in a considerably more advanced state of chondrification than in the previous stage. The pila occipitalis has increased in height. Three hypoglossal foramina are present on one side while four are visible on the dorsal surface of the other side (Figs. 3 and 4), the anterior two being foramina for the twigs of the anterior hypoglossal component. Anterior to these two twigs of the anterior hypoglossal root a third twig is still present. It does not join the other twigs as in the previous stage but

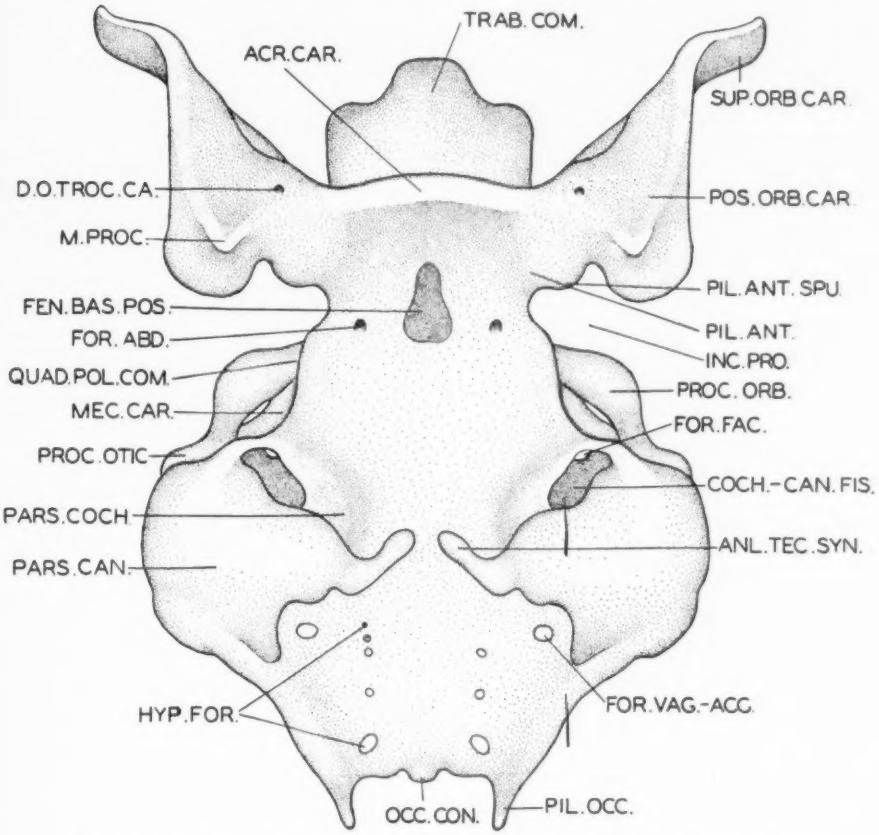

Fig. 3 (× = 40.8). Stage 2. Reconstruction of chondrocranium (norma dorsalis).

merely penetrates the dorsal surface of the basal plate for a short distance and then disappears. The cranial ribs have disappeared.

A fenestra basicranialis posterior is present in the anterior region of the basal plate (Figs. 3, 4, and 5). It has been formed by the resorption of intercellular substance above and below the chorda. The dorsal area of resorption is not situated directly above the ventral area but some distance anteriorly (Fig. 4), so that when seen from above the fenestra appears as a depression on the dorsal surface of the basal plate (Fig. 3). Formation of the fenestra basicranialis posterior by resorption of the basal plate has been described in *Tinnunculus* only (SUSCHKIN, 1899). In the duck (SONIES, 1907, DE BEER and BARRINGTON, 1934) and fowl it is formed between the medial edges of the parachordals. *Spheniscus* (CROMPTON, 1953) and *Struthio* (FRANK, 1954) lack a posterior basicranial fenestra.

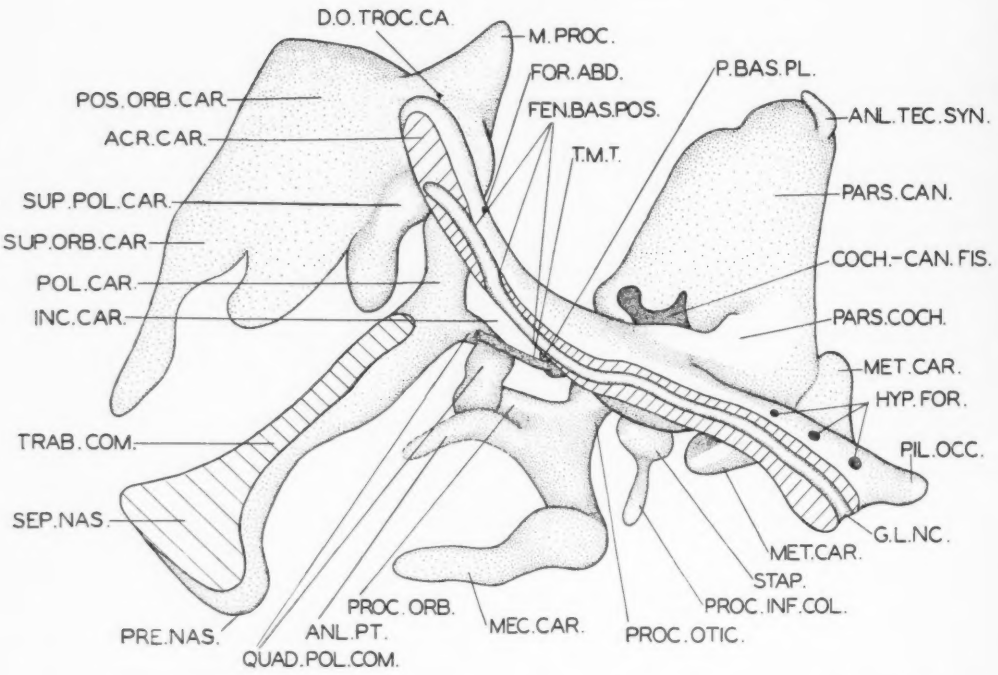

Fig. 4 (× = 48.8). Stage 2. Disposition of cranial entities as viewed from a median sagittal section.

Broadening of the basal plate between the otic capsule and the root of the pila antotica has enclosed the abducens in a foramen (Figs. 3 and 4). No trace of a lateral abducent commissure participating in the formation of the foramen for the abducens as described by CROMPTON (1953) in the penguin, could be demonstrated for *Pyromelana*. Progressive deposition of intercellular substance in the undifferentiated tissue lateral to the posterior part of the orbital cartilage has increased the size of this structure, so that it now encloses the trochlear in a long canal. The dorsal orifice of this canal is shown in Figs. 3 and 4, and the ventral orifice in Figs 5 and 6. A similar canal for the trochlear has been described in *Pygoscelis papua* (PARSONS, 1932), *Spheniscus demersus* (CROMPTON, 1953) and *Struthio* (FRANK, 1954). Postero-lateral to the dorsal aperture of the trochlear canal the posterior part of the orbital cartilage bears a cone-like projection of mesenchymatous tissue containing some intercellular deposit (Figs. 3, 4 and 6). This structure is of transitory nature and disappears in later stages.

An incipient pila antotica spuria is present as a short blastematous outgrowth from the ventral edge of the posterior part of the orbital cartilage (Fig. 3). The profundus branch of the trigeminal is lodged in a shallow fissure between

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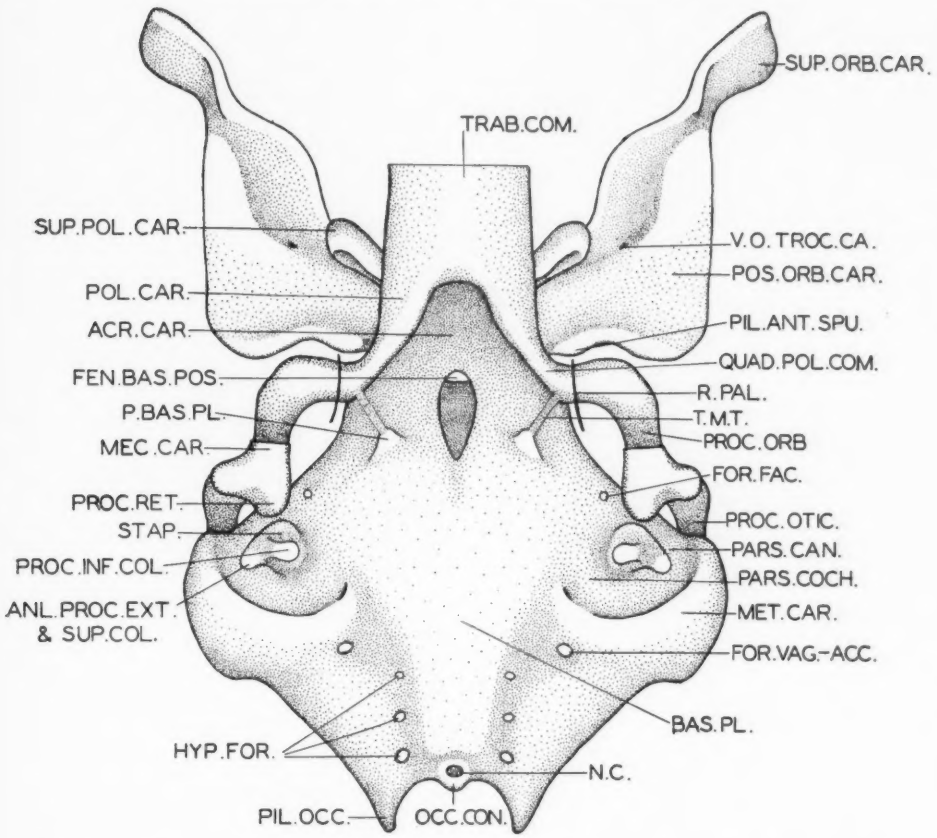

Fig. 5 (× = 40.8). Stage 2. Reconstruction of the posterior part of the chondrocranium (norma ventralis).

the medial edge of the pila antotica spuria laterally, the ventral edge of the posterior part of the orbital cartilage dorsally and the lateral edge of the base of the pila antotica medially. The antero-lateral corner of the posterior part of the orbital cartilage extends anteriorly over the eye-ball as a thin plate of mesenchymatous tissue containing some intercellular substance. This part can be referred to as the supraorbital part of the orbital cartilage. (Figs. 3, 4, 5 and 6).

The medial anlage of the metotic cartilage has fused with the lateral anlage and with the more medial part of the postero-lateral wall of the pars canalicularis (Figs. 3, 5 and 6). Fusion has also taken place between the lateral edge of the basal plate and the medial surface of the medial anlage of the metotic cartilage anterior to the point at which the vagus-accessory nerve leaves the cranial cavity. In this manner part of the fissura metotica has been enclosed

as a foramen for the nerve (Figs. 3 and 5). The glossopharyngeal leaves the cranial cavity through the anterior corner of the remaining part of the fissura metotica. The cochleo-canalicular fissure, confluent with the incisura prootica in the previous stage, has now been delimited anteriorly by the fusion of the anterior walls of the cochlear and the canalicular portions of the otic capsule. This cartilaginous bridge in front of the facial is not homologous with the prefacial commissure of reptiles because it is not situated between the otic capsule and the lateral edge of the basal plate but between the cochlear and canalicular portions of the otic capsule.

With the increase in height of the pars canalicularis and the development of a medio-dorsal wall for the lateral part of the otic capsule, the large vacuity between the medial edge of the roof of the pars canalicularis and lateral edge of the roof of the pars cochlearis has diminished in size. The postero-dorsal corner of the pars canalicularis bears the anlage of the tectum synoticum as a medially directed procartilaginous process, connected to its companion on the opposite side by a faint tract of mesenchymatous tissure (Fig. 3).

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2 *The Anterior End of the Chondrocranium*

The paired trabecular anlagen have elongated and fused to form a median trabecula communis. In this manner the hypophysial incisure has been converted into a fenestra hypophyseos, bordered posteriorly by the anterior surface of the acrochordal, postero-laterally by the polar cartilages and antero-laterally by the diverging posterior ends of the trabecula communis. In the region between the nasal sacs the trabeculae are completely fused, but in the orbital region the double nature of the trabecula communis can still be detected. In transverse sections the trabeculae can be seen as two lateral centres of histologically older procartilage, and between them, there is a thin strip in which the cells are less densely packed and the amount of intercellular deposit is less.

In the previous stage the distance between the medial edges of the trabecular anlagen measured 180μ, and the diameter of each anlage was 210μ. This gives a maximum width of 600μ between the lateral edges of the trabeculae. In the present stage the diameter of the trabecula communis measures only 450μ. This leaves the layer of procartilage between the trabeculae with a maximum width of 30μ. Actually it is even less than that, because the diameter of the trabeculae has increased.

These measurements clearly show that the trabeculae converge towards the midline to form the trabecula communis without the intervention of a separate intertrabecula as claimed by the PARKERS and SUSCHKIN (1899).

Between the nasal sacs the internasal septum has appeared as an upgrowth of the dorsal edge of the trabecula communis. The nasal region of the trabecula communis is at an angle with the orbital region (Figs. 4 and 6). The base of

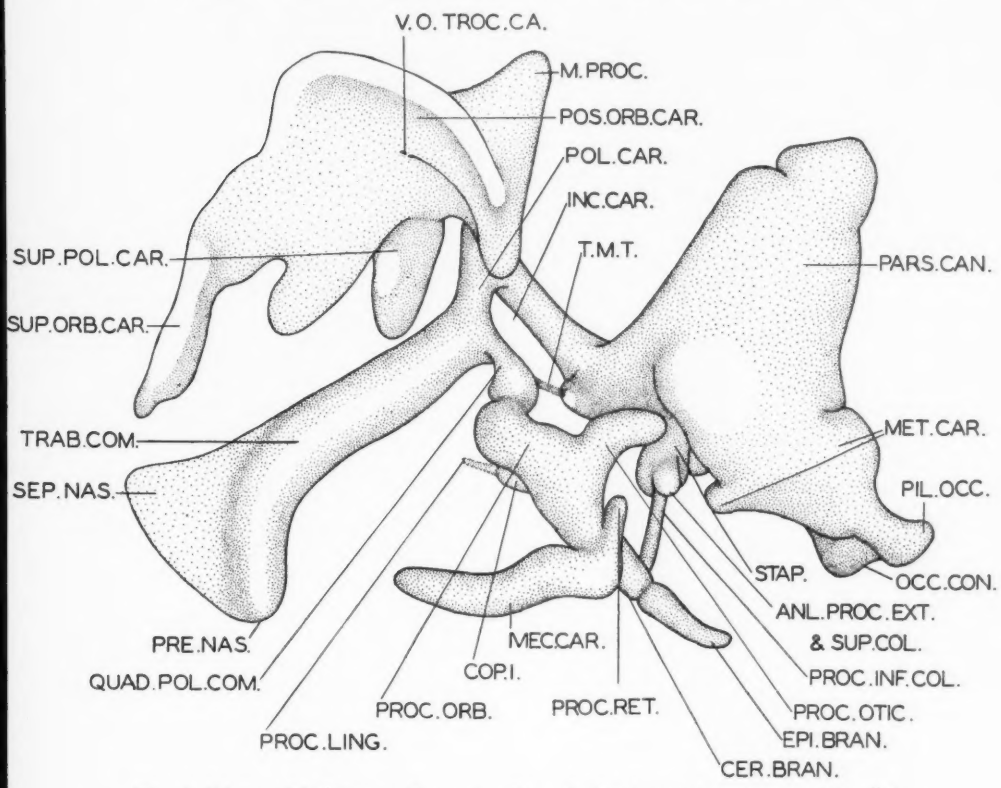

Fig. 6 (×=40.8.) Stage 2. Reconstruction of chondrocranium (norma lateralis).

the septum (which will be referred to in this paper as the trabecular bar), is in a more advanced state of chondrification than the rest of the septum.

The procartilaginous anterior tip of the trabecular bar projects anteriorly beyond the nasal sacs as a short prenasal process (Figs. 4 and 6).

This structure becomes a very conspicuous feature of the chondrocranium in later stages. The olfactory nerves are very short and run downwards and slightly backwards along the lateral surfaces of the septum nasi to the nasal sacs situated in this stage beneath the cerebral hemispheres.

In the orbital region there is very little upgrowth of the trabecula communis. It is still possible to distinguish histologically between the polar cartilages and trabecula communis. Fusion between polar cartilages and acrochordal is complete. The suprapolar has chondrified. Its ventral edge is still free, so that the relations of the ophthalmic artery are as in the previous stage. The postero-ventral corner of the polar cartilage is in procartilaginous continuity with the pars quadrata. A more detailed description of this will be given under the visceral arch skeleton.

The cranial flexure is less pronounced than in the previous stage; the angle between the basal plate and longitudinal axis of the polar cartilage measuring $\pm 47°$ compared to $\pm 45°$ in the previous stage, and $\pm 101°$ between the basal plate and longitudinal axis of the trabecula communis compared to $\pm 97°$ in the previous stage. There has been no change in the angle between the longitudinal axis of the polar cartilage and the trabecula communis, indicating that the trabeculo-polar bar rotates as a whole.

3 *The Visceral Arches*

Meckel's cartilage has in the meantime developed very rapidly and projects antero-medially from the base of the pars quadrata (Figs. 3, 4 and 6). The retro-articular process has elongated (Fig. 6). A processus oticus has developed as an outgrowth on the postero-dorsal corner of the pars quadrata. It projects postero-laterally towards the antero-ventral wall of the canalicular part of the otic capsule (Figs. 3, 4, 5 and 6). At this stage the tip of the pr. oticus is still separated from the capsular wall, and articulation with the latter only takes place in later stages. Attention has already been called to the procartilaginous continuity existing between the orbital process of the pars quadrata and the polar cartilage. The medial tip of this quadratopolar commissure (as this structure will be referred to in this paper) is fused to the antero-ventral corner of the polar cartilage (Figs. 4, 5 and 6); and its distal tip joins on to the medial surface of the processus orbitalis a short distance behind its anterior tip (Figs. 3, 5 and 6). It passes dorsal to the palatine nerve. FILATOFF (1906) described a procartilaginous continuity between the quadrate and trabecula in the pigeon. (He did not observe the polar cartilages). DE BEER and BARRINGTON (1934) found a similar band of procartilaginous tissue stretching between the quadrate and the polar cartilage and considered it as a remnant of the basal articulation. In *Pyromelana* a procartilaginous quadratopolar commissure was found on both sides in three embryos.

Underlying the internal carotid artery is a thin tract of mesenchymatous tissue containing some intercellular deposit. It stretches between a procartilaginous protuberance on the basal plate to the quadratopolar commissure (Figs. 4, 5 and 6) and is attached to the medial surface of the latter a short distance behind the fusion of its medial tip to the polar cartilage.

The connexion between the polar cartilage and the pars quadrata is apparently established by the elongation in a medio-dorsal direction of the procartilaginous process on the medial surface of the processus orbitalis of the previous stage. In an embryo that, in several features, has reached the same degree of development as the present stage there occurs what seems to be an intermediate condition between stages 1 and 2 as far as the development of this commissure is concerned. In this embryo the procartilaginous process on the medial

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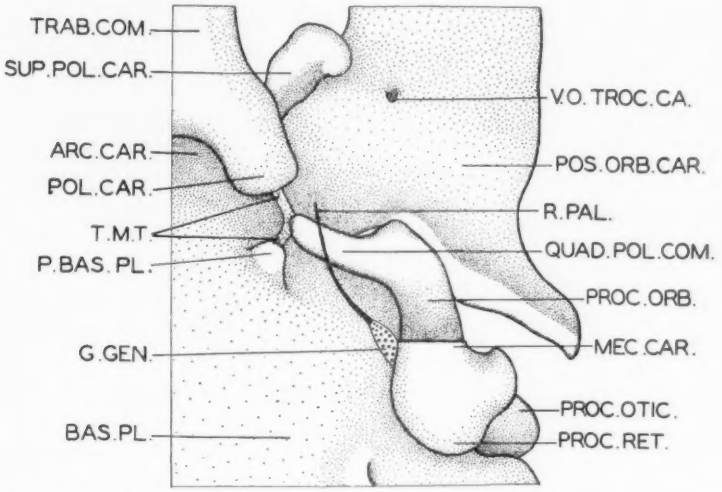

Fig. 7 (× = 45.4). Intermediate stage. Reconstruction of chondrocranium (norma ventralis) showing the topography in the region of the quadratopolar commissure.

surface of the processus orbitalis is elongated above the palatine nerve and terminates a short distance ventro-lateral to the antero-ventral corner of the polar cartilage (Fig. 7). Thin strands of mesenchymatous tissue connect the tip of this process with the antero-ventral corner of the polar cartilage and with the protuberance on the ventral surface of the basal plate. The latter connexion underlies the internal carotid artery.

Attention may here be drawn to the relation of the mesenchymatous anlage of the pterygoid to the pars quadrata. The pterygoid is the first dermal bone to appear in the skull of *Pyromelana*, and its mesenchymatous anlage is also the first to arise. In this stage it is present as a mesenchymatous rod, the posterior end of which is confluent with the medial surface of the pars quadrata at the base of the orbital process (Fig. 4). It projects antero-medially towards the midline. The pterygoid anlage, when it first appears, already shows the typical "neognathic" condition of "carinate" birds. At this stage the posterior end of the pterygoid anlage is in continuity with the pars quadrata and the articulation between the posterior end of the pterygoid ossifying in this anlage, and the pars quadrata, develops later.

The mesenchyme cells of the extracollumellar part of the columella auris are much more concentrated than in the previous stage so that this region is relatively much smaller, than in the previous stage, and it is now more clearly demarcated. The pr. infracolumellaris contains a procartilaginous core. Continuity of intercellular substance still exists between the medial tip of the stapedial part and the cochlear floor. The greater affinity for Bismarck brown shown by the

intercellular deposit in the former indicates a greater concentration of intercellular deposit than in the cochlear floor.

A procartilaginous epibranchial has differentiated in the distal mesenchymatous portion of the anlage of the first branchial arch (Fig. 6). It is separated from the proximal cartilaginous element, the ceratobranchial, by a zone of mesenchymatous tissue. In the median copular anlage two elements have chondrified. The ventral tip of the ceratobranchial is connected to the antero-lateral corner of copula 2. Near its posterior end the lateral surface of copula 1 bears a short postero-laterally directed process; "the lateral wing of the copula", (KALLIUS, 1905). CROMPTON (1953) also found this process in the penguin. The anterior end of copula 1 is drawn out as a thin processus lingualis.

C STAGE III

Total length of embryo: 25 mm.

1 *Basal Plate and Otic Capsules*

Further absorption of the basal plate dorsal to the chorda has increased the size of the fenestra basicranialis posterior, which is closed by a membrane and traversed by the chorda. Dorsal to the tip of the chorda the acrochordal shows signs of resorption. Only three hypoglossal foramina are now present on each side of the basal plate.

A conspicuous feature of the otic capsule at this stage is the posterior rotation of the pars canalicularis. (Compare figs. 6 and 8). This rotation results in the pars canalicularis occupying a position postero-dorsal to the pars cochlearis, and the tectum synoticum sloping upwards and backwards. All the walls of the otic capsule except the medial wall and part of the cochlear floor in the vicinity of the medial end of the columella auris are now more or less chondrified. The large vacuity in the medial wall has been bridged over by a procartilaginous commissure, dividing it into an anterior foramen for all the branches of the acustic nerve and a postero-dorsal for the endolymphatic duct. Intercellular deposit has appeared in the mesenchymatous tissue separating the facial nerve from the cavity of the otic capsule.

In the lateral part of the procartilaginous anlage of the metotic cartilage the latter has begun to chondrify as a cartilaginous plate underlying the floor of the canalicular part of the otic capsule. Its dorsal surface is however still attached to the floor of the latter by procartilaginous tissue. The free postero-dorsal edge of the procartilaginous anlage of the metotic cartilage has increased in height, and medially it passes over indistinguishably into the pila occipitalis. (Compare figs. 6 and 8).

A foramen perilymphaticum has developed in the floor of the otic capsule and opens postero-ventrally into the anterior end of the fissura metotica.

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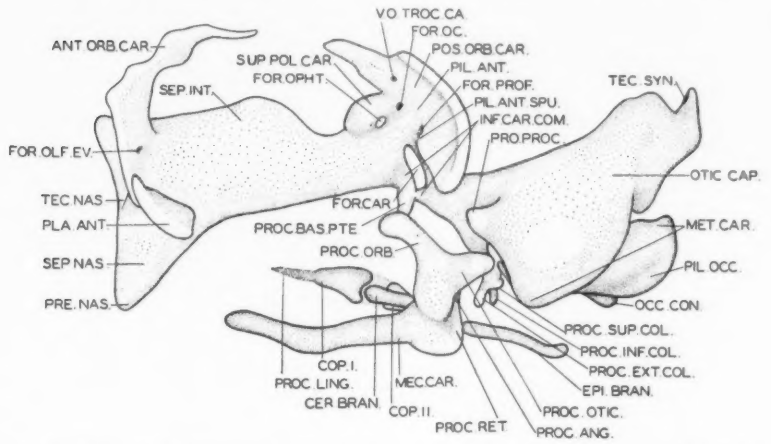

Fig. 8 (×=21.5). Stage 3. Reconstruction of chondrocranium (norma lateralis).

A cavity, the recessus scalae tympani, thus formed, is bordered latero-dorsally by the lateral edge of the foramen perilymphaticum, medio-dorsally by the medial edge of the foramen perilymphaticum and medially by the lateral edge of the basal plate. A floor to the posterior part of the recessus scalae tympani is formed by the commissure between metotic cartilage and the basal plate anterior to the foramen for the vagus-accessory nerve (Fig. 9). Antero-dorsally the recessus scalae tympani communicates with the cavity of the otic capsule, medially with the cranial cavity through the apertura medialis recessus scalae tympani, posteriorly with the cavum metoticum between the floor of the pars canalicularis and medial part of the metotic cartilage; and finally antero-laterally to the exterior through the apertura lateralis recessus scale tympani. At this stage all these cavities are still filled with embryonic tissue. Lateral to the recessus scalae tympani the cavum metoticum has not yet developed.

The foramen perilymphaticum has developed through resorption of intercellular substance in the floor of the otic capsule approximately on the boundary between cochlear and canalicular portions. In the previous stage the area in which the foramen perilymphaticum was to develop was procartilaginous like the rest of this region. Judging from CROMPTON'S (1953) description the foramen perilymphaticum in the penguin is a remnant of the cochleo-canalicular fissure.

On the anterior wall of the pars canalicularis an anteriorly directed prootic process has developed (Fig. 8). This structure has thus far only been recorded by SONIES (1907) for the duck and fowl, by DE BEER and BARRINGTON (1934) for the duck, and by FRANK (1954) for the ostrich.

A procartilaginous posterior part of the orbital cartilage has now developed in the large diffuse anlage of previous stages. (Compare figs. 6 and 8). There

is still a lot of diffuse tissue containing faint traces of intercellulur deposit attached to the lateral part of the postero-dorsal surface of the posterior part of the orbital cartilage, but this has been left out in the reconstruction. Its postero-ventral corner has expanded laterally and ventrally, and the ventral end of the now procartilaginous pila antotica spuria has fused to the lateral edge of the basal plate enclosing the profundus nerve in a foramen (Figs. 8 and 9). The remaining branches of the trigeminal nerve leave the cranial cavity through the incisura prootica.

2 *The Anterior End of the Chondrocranium*

The prechordal region is now more firmly attached to the acrochordal and basal plate, and the cranial axis has straightened out considerably. (Compare figs. 6 and 8). The angle between orbital and nasal regions persists.

An interorbital septum has developed behind the nasal region as an upgrowth of the trabecula communis. Its posterior portion remains rather low (Fig. 8), but anterior to the optic nerves it suddenly increases in height. The anterior part of the orbital cartilage is attached to the lateral surface of the interorbital septum some distance below its dorsal edge. The olfactory nerve leaves the cranial cavity through the foramen olfactorium evehens (Fig. 8) bordered laterally by the ventral edge of the anterior part of the orbital cartilage and medially by the lateral surface of the interorbital septum. The dorsal edge of the interorbital septum projects some distance anterior and dorsal between the ventral edges of the anterior parts of the orbital cartilage (Fig. 8).

Examination of series of transverse sections of embryos intermediate in development between the previous and present stages shows how the development of this region takes place. The anterior part of the orbital cartilage develops by the deposition of intercellular substance in the layer of undifferentiated mesenchymatous tissue underlying the brain.

More posteriorly the supraorbital and posterior parts of the orbital cartilage also appear in this layer. Its development proceeds from the dorsal edge of the interorbital septum outwards so that it appears as a process of the latter. It is connected to the septum in such a way that the olfactory nerve is enclosed in a foramen bordered laterally by its ventral edge, and medially by the dorsal edge of the septum. This foramen through which the olfactory nerve leaves the cranial cavity is the foramen olfactorium evehens. It occupies a similar position in *Tinnunculus* (Suschkin, 1899) and *Gallus* (Gaupp, 1906), and Sonies, (1907). Sonies (1907), and de Beer and Barrington (1934) describing the development of *Anas*, and Crompton (1953) in his description of the development of *Spheniscus*, do not mention the position of the olfactory nerve relative to the attachment of the anterior part of the orbital cartilage and the interorbital septum in the early stages, but judging from their descriptions of older em-

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bryos, the olfactory nerve must be situated anterior to this attachment. In those forms the foramen olfactorium evehens is formed later in ontogeny by the development of a sphenethmoid commissure between the posterior edge of the parietotectal and the anterior part of the orbital cartilage.

When the anterior parts of the orbital cartilages can be observed for the first time as structures containing some intercellular deposit, they are situated above the posterior portions of the nasal sacs, and the olfactory nerves run straight downwards through the foramina olfactoria evehentia to the nasal sacs. At that stage of the development there is no upgrowth of the dorsal edge of the septum between the anterior parts of the orbital cartilages. Further increase in height of the interorbital septum behind the nasal sacs carries the anterior orbital cartilages upwards. The foramen olfactorium evehens is thus shifted dorsally, and the condition obtaining in the present stage is reached. The olfactory nerve has elongated in the meantime, and after leaving the cranial cavity through the foramen olfactorium evehens, it pursues an antero-ventral course along the interorbital septum before reaching the nasal sac. Growth also occurs along the dorsal edge of the interorbital septum in this region so that in the present stage the dorsal edge projects some distance anterior and dorsal to the junction between the anterior part of the orbital cartilage and the interorbital septum (Fig. 8). The anterior part of the orbital cartilage projects postero-laterally over the eye-ball, and a strand of connective tissue stretches between its postero-lateral corner and the antero-lateral corner of the posterior part of the orbital cartilage; this strand lies in the position occupied by the supraorbital part of the orbital cartilage in the previous stage.

Although some embryos of more or less the same age were examined, it was not possible to find one in which a closed sphenoid fontanelle was present as is the case in *Anas* (DE BEER and BARRINGTON, 1934), *Spheniscus* (CROMPTON, 1953), *Ardea* (SLABÝ, 1952) and *Struthio* (FRANK, 1954). If such a stage does occur in the development of *Pyromelana*, it must be of very brief duration, and possibly in the procartilaginous stage. In contrast with the forms mentioned above the orbital cartilages in *Pyromelana* are very thin.

In the nasal region a tectum nasi has now appeared above the posterior region of the nasal sac (Fig. 8). The anterior part of this roof is in the form of a laterally directed ridge of dense mesenchymatous tissue (not included in the reconstruction) along the procartilaginous dorsal edge of the septum nasi. More posteriorly this ridge increases in breadth, and its lateral edge has begun to curl downwards. Some intercellular substance is already present in this region of the tectum nasi. Examination of transverse sections of intermediate stages shows that the deposition of intercellular material proceeds from the dorsal edge of the septum nasi outwards into mesenchymatous ridges.

A planum antorbitale has developed behind the nasal sac (Fig. 8). It is a

vertical procartilaginous plate situated anterior to the eye-ball and postero-lateral to the hind end of the nasal sac.

Its medio-dorsal corner is in blastematous connexion with the posterior edge of the tectum nasi. Between the medial edge of the planum antorbitale and the lateral surface of the interorbital septum is a wide fissura orbitonasalis through which the profundus and olfactory nerves enter the nasal capsule. Laterally the lateral edge of the planum antorbitale projects far beyond the tectum nasi and the nasal sac. Behind the planum antorbitale the dorsal edge of the interorbital septum forms a lateral ridge-like outgrowth (Fig. 8), which will develop into a prominent structure in later stages.

Posteriorly the prechordal region on each side has acquired two additional connexions with the chordal region besides those present between the polar cartilages and the anterior surface of the acrochordal. The ventral edge of the suprapolar cartilage has fused with the dorsal edge of the polar cartilage and with the dorsal edge of the posterior portion of the trabecula immediately in front of it, thus enclosing the ophthalmic artery between them. Apparently the foramen is formed only between polar and suprapolar cartilages, the posterior part of the trabecula taking no part in its formation. The postero-dorsal edge of the suprapolar cartilage has fused to the pila antotica for some considerable distance. The line of demarcation between the two structures is indicated by the ventral orifice for the canal of the oculomotor nerve (Fig. 8).

Below the internal carotid artery further deposition of intercellular substance in the strand of tissue described in the previous stage has resulted in the establishment of a cartilaginous commissure between the polar cartilage and the basal plate (Figs. 8 and 9). In this manner the incisura carotica of previous stages has been converted into a foramen caroticum (Fig. 8). For reasons to be explained in the discussion, the commissure between the basal plate and the polar cartilage will be referred to as the infracarotid commissure in this paper.

Resorption of intercellular substance in the quadratopolar commissure has taken place some distance lateral to the point where the palatine nerve is continued forwards underneath this structure. The dorsal part is chondrified in continuity with the polar cartilage. Its basal portion projects postero-ventrally and slightly laterally beneath the basal plate, while the more distal part projects latero-ventrally and slightly anteriorly (Figs. 8 and 9). The whole of this dorsal part of the commissure, fused to the polar cartilage, must be considered as the processus basipterygoideus. It is entirely of visceral origin as no outgrowth of the polar cartilage partakes of its formation. Its distal tip is syndesmotically connected to a procartilaginous remnant of the quadratopolar commissure on the medial surface of the pr. orbitalis. This will be described further under the visceral arches.

Returning now to the ventral border of the foramen caroticum, it is seen that its anterior part is formed by the proximal part of the pr. basipterygoideus,

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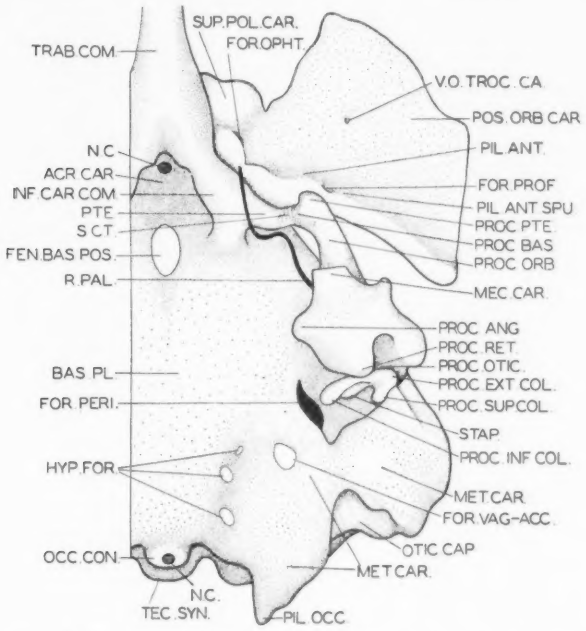

Fig. 9 ($\times = 33.1$). Stage 3. Reconstruction of the posterior part of the chondrocranium (norma ventralis).

its posterior by the protuberance on the ventral surface of the basal plate and its median by chondrification in the strand of mesenchymatous tissue streching between the medial surface of the proximal part of the pr. basipterygoideus and the protuberance of the basal plate.

3 *The Visceral Arches*

Meckel's cartilage has elongated. (Compare figs. 6 and 8). The retro-articular process has grown farther posteriorly, and on the medial edge a processus angularis has started to grow out (Figs. 8 and 9). The first indications of a future articulation between the lower end of the pars quadrata and Meckel's cartilage are present. The dorsal end of the processus oticus is still separated from the auditory capsule. With the resorption of intercellular substance in the quadrato-polar commissure a small stump has remained on the medial surface of the orbital process of the pars quadrata; it is the basal process of the pars quadrata, and the tip of the processus orbitalis projecting anterior to it can be considered as the processus pterygoideus.

The columella auris has now chondrified. Of special interest is the relation of the proximal end of the stapedial part of the floor of the pars cochlearis. The

proximal end has enlarged to form the footplate of the future stapes. It is well chondrified and embedded in the capsular floor. Although the rest of the floor of the otic capsule has chondrified, that part in the vicinity of the proximal end of the columella auris has remained procartilaginous, and the zone immediately around the enlarged proximal tip contains less intercellular substance than in the previous stage. There is a great difference in the amount of intercellular substance in the two structures, and the cartilage of the footplate is easily distinguishable from that of the otic capsule. Its cartilage lacunae are very large, often containing more than one nucleus. The concentric arrangement of chondrocytes of the capsular wall around the footplate gives the impression that the latter was pushed into the capsular wall.

Postero-dorsally the extracolumellar part bears the anlage of the pr. supracolumellaris in the form of a mesenchymatous protuberance (Figs. 8 and 9). Lateral to this anlage the rest of the extracolumellar part projects as the pr. extracolumellaris. It was not possible to ascertain whether this anlage of the processus supracolumellaris develops as an outgrowth of the extracolumellar part of the columella auris subsequent to chondrification of the latter, or whether it is part of the original diffuse anlage of this part of the columella auris.

In the branchial arch the epibranchial is now properly chondrified but it is still less differentiated than the ceratobranchial, which latter has lost its former continuity with copula 2. Faint traces of the intercellular deposit is present in the zone of mesenchymatous tissue between the copulae. The lateral wings of copula 1 have disappeared.

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D STAGE IV

Total length of embryo: 30 mm.

1 *The Basal Plate and Otic Capsules*

More resorption of the basal plate and acrochordal has taken place in the vicinity of the chorda. Except where the latter pierces the occipital condyle, all intercellular substance above it has been resorbed. This regression of the chondrocranium is even more marked anterior to the fenestra basicranialis posterior where the basal plate and, more anteriorly, the ventral part of the acrochordal has become so thin that the chorda is situated above their dorsal surfaces. The middle portion of the acrochordal dorsal to the tip of the chorda has been completely resorbed, its place being occupied by connective tissue. The cartilaginous posterior wall of the hypophysial pit thus shows a notch above the tip of the chorda (Fig. 10). Behind the fenestra basicranialis posterior reduction of the basal plate has only taken place immediately above the chorda so that the latter now lies in a groove extending from the fenestra basicranialis posterior to the

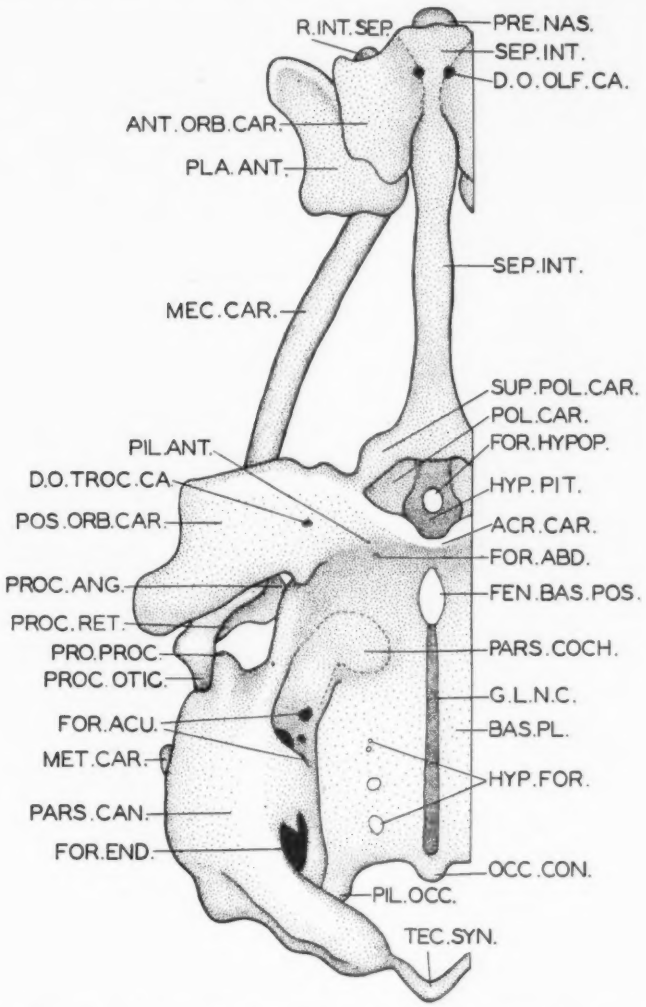

Fig. 10 ($\times = 25.2$). Stage 4. Reconstruction of chondrocranium (norma dorsalis).

anterior end of the occipital condyle (Fig. 10). This groove is covered by a thin membrane.

On the one side the two posterior hypoglossal roots leave the cranial cavity through their respective foramina. The anterior root has two foramina on the dorsal side (Fig. 10) but emerges through a single foramen on the ventral side of the basal plate. On the other side the anterior root has two separate foramina, a small anterior foramen running obliquely backwards and opening close to the posterior one on the ventral surface of the basal plate. The two twigs of the anterior hypoglossal root unite immediately after emerging from their foramina.

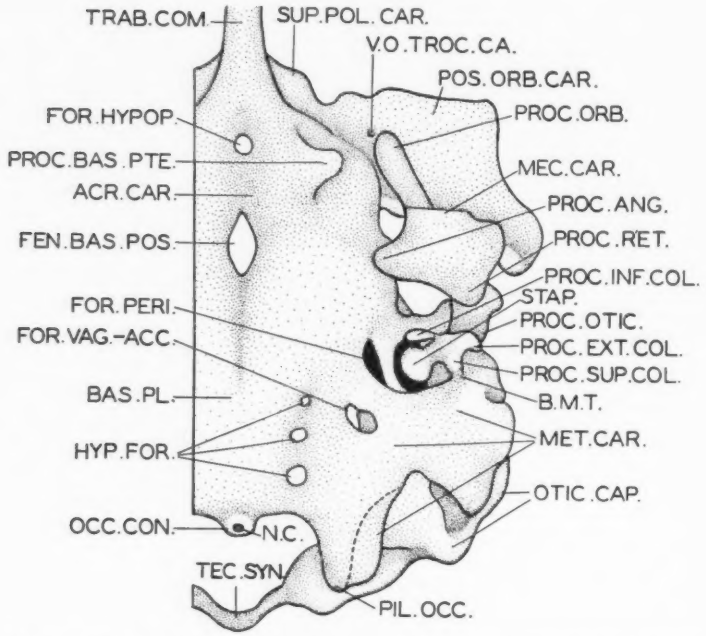

Fig. 11 ($\times = 25.2$). Stage 4. Reconstruction of the posterior part of the chondrocranium (norma ventralis).

The otic capsule is now properly chondrified except for some procartilaginous patches in its medial wall. The canalicular part has expanded laterally, medially and especially posteriorly so that it is now situated more or less behind the cochlear part. This can be seen in Fig. 10 where the outline of the cochlear cavity is indicated by a dotted line. In a wax model the prominences of the semicircular canals can already be clearly made out. The tectum synoticum is procartilaginous for its whole length. The foramen endolymphaticum is still a wide aperture (Fig. 10), but the large anterior foramen of previous stages has been divided into four smaller ones for the different branches of the acustic nerve. These foramina are all situated in a depression on the medio-dorsal surface of the otic capsule on the boundary between the cochlear and the canalicular divisions (Fig. 10).

The anterior wall of the canal of the facial nerve has increased in thickness (Fig. 10), and its medial edge has spread on to the lateral edge of the basal plate in front of the anterior boundary of the cochlear part of the otic capsule. This most anterior part of the commissure in front of the facial nerve is thus comparable to the prefacial commissure in other forms.

Beneath the pars canalicularis the metotic cartilage has chondrified as a thick plate and is separated from the capsular floor by a space filled with connective

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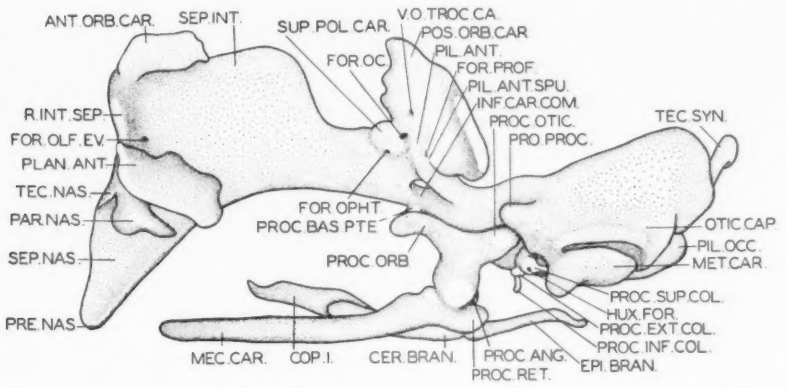

Fig. 12 (× = 19.5). Stage 4. Reconstruction of chondrocranium (norma lateralis).

tissue. It has been formed by the resorption of intercellular substance from the procartilaginous tissue occupying this position in previous stages. Antero-laterally the metotic cartilage is connected to the pars canalicularis in the region of the ampulla of the lateral semicircular canal (Figs. 11 and 12). For the rest the anterior and lateral edges are free from the otic capsule. (Fig. 11). The posterior region of that part of the metotic cartilage which has developed from the median anlage is still procartilaginous. (The outline of this region is indicated by a dotted line in Fig. 11). Its posterior edge is in procartilaginous continuity with the otic capsule.

2 *The Anterior End of the Chondrocranium*

When figures 8 and 12 are compared the development that has taken place in this region is evident. The interorbital septum has increased in height anterior to the optic nerves, and the whole ethmoidal region has undergone considerable enlargement. There is no longer a definite angle between the bases of the orbital and the ethmoidal regions as in the previous stages but the base of the whole prechordal region is now arched (Fig. 12).

In the ethmoidal region the procartilaginous anlage of the tectum nasi has chondrified as the parietotectal cartilage. Anterior to it a lateral mesenchymatous outgrowth (not included in the reconstruction), already present in the previous stage, has extended farther forwards. The lateral edge of the parietotectal cartilage curls downwards to form the side wall of the nasal capsule (Fig. 12), and inwards to form the maxilloturbinal cartilage. The latter structure is still procartilaginous at this stage. At its posterior end the dorsal surface of the maxilloturbinal is connected by means of mesenchymatous tissue to the anterior surface of the planum antorbitale near its latero-ventral corner. The blastema-

tous connexion between the paries nasi and the planum antorbitale has chondrified.

The planum antorbitale has increased in size along all its edges. Its medial edge has grown up in a medio-dorsal direction narrowing the orbitonasal fissure and carrying it towards the foramen olfactorium evehens. (Compare figs. 8 and 12). In this stage, then, the olfactory nerve transverses the orbit for a shorter distance before it enters the nasal capsule. The ventral part of the medial edge of the planum antorbitale approaches the interorbital septum more closely than does the dorsal part (Fig. 12). This wide dorsal part of the fissure between the medial edge of the planum antorbitale and septum, through which the olfactory and the profundus branch of the trigeminal nerve enter the nasal capsule, can be distinguished as the foramen olfactorium advehens. It is bordered anteriorly by the posterior edge of the tectum nasi, medially by the lateral surface and incompletely ventrally by the medial edge of the planum antorbitale. Another noteworthy feature of the ethmoidal region is that the trabecular bar has increased in diameter and is much thicker than the portion of the septum above it.

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The orbital cartilages have been subjected to much regression. (Compare figs. 8 and 12). The whole of the structure above the eye and a large part of the region anterior to it have been resorbed. Some resorption has also taken place at the anterior end of the posterior part of the orbital cartilage. What remains of the orbital cartilage, however, is now thoroughly chondrified.

The ventral portion of the anterior part of the orbital cartilage projecting latero-dorsally from the septum in the previous stage has become flattened against and fused to the lateral surface of the upgrowing interobital septum. This rotation of the ventral portion of the anterior part of the orbital cartilage, and the fusion of its medial surface to the lateral surface of the interorbital septum can be followed very accurately in transverse series. The result of all this is that above the foramen olfactorium evehens the olfactory nerve has become enclosed in an olfactory canal situated between the lower portion of the anterior part of the orbital cartilage and the interorbital septum. The free dorsal portion of the anterior part of the orbital cartilage projects latero-dorsally from the dorsal edge of the interobital septum (Figs. 10 and 12). The anterior edge of the ventral portion of the anterior part of the orbital cartilage is fused to the ventral surface of a ridge-like lateral outgrowth along the dorsal edge of the anterior part of the interorbital septum. Near its ventral end the olfactory canal is bordered only by the orbital cartilage and the interorbital septum but more dorsally the nerve is situated more towards the dorsal edge of the interorbital septum. In this region the canal is bordered laterally by the orbital cartilage, medially by the interorbital septum and dorsally by the outgrowth along the dorsal edge of the interorbital septum. The dorsal reconstruction (Fig. 10), in which the outline of the anterior part of the orbital cartilage is indicated by

a dotted line, shows the positions of the various entities around the dorsal orifice of the olfactory canal. In transverse sections it is easy to distinguish between the different entities concerned on account of their histological differences. Not only are the chondrocytes in the orbital cartilage more densely packed and more rounded in appearance, but the intercellular substance stains much more deeply with Bismarck brown than in the interorbital septum.

The cartilaginous entities bordering the hypophysial pit laterally have so completely fused that it is impossible to trace original outlines. The only clue to their position is the position of the foramina enclosed between them at the time of fusion. A more rigid connexion has been established between the basal plate and the prechordal region by a medial extension of the infracarotid commissure. It is not impossible that anteriorly a medial outgrowth of the polar cartilage also partakes in broadening this commissure. The result of this broadening of the infracarotid commissure is that the internal carotid artery enters the hypophysial pit through an antero-dorsally directed canal (Fig. 12).

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3 *The Visceral Arches*

Meckel's cartilage has grown forwards and approaches the tip of the prenasal process much more closely than in the previous stage. (Compare figs. 8 and 12). Posteriorly separation between the lower end of the pars quadrata and Meckel's cartilage has continued further, and the two structures are nearly separated except for a lateral bridge. The procartilaginous pr. basalis as well as the syndesmotic connexion between the latter and the tip of the pr. pterygoideus have disappeared. The pr. orbitalis of the pars quadrata is now widely separated from the tip of the pr. basipterygoideus; the anlage of the pterygoid, already containing an osseous core, is situated between them but there is no connexion between this anlage and the distal tip of the pr. basipterygoideus.

Intercellular substance has been deposited in the anlage of the pr. supracolumellaris leaving a small area at its base devoid of any deposit. (Fig. 12.) This is "Huxley's foramen" or foramen intracolumellare (SONIES, 1907). A band of mesenchymatous tissue connects the tip of the pr. supracolumellaris with the ventral surface of the metotic cartilage. The zone of the capsular floor surrounding the footplate of the columella auris has lost all its intercellular substance, thus forming a foramen ovale into which the footplate fits (Fig. 11). Between the equator of the latter and the edges of the fenestra ovalis a dense layer of connective tissue is present. A similar layer, the remnant of the capsular wall medial to the footplate, separates the medial surface of the latter from the cavity of the otic capsule.

The first indication of the future fusion between the ceratobranchial and the epibranchial is present as a slight deposition of intercellular substance in the

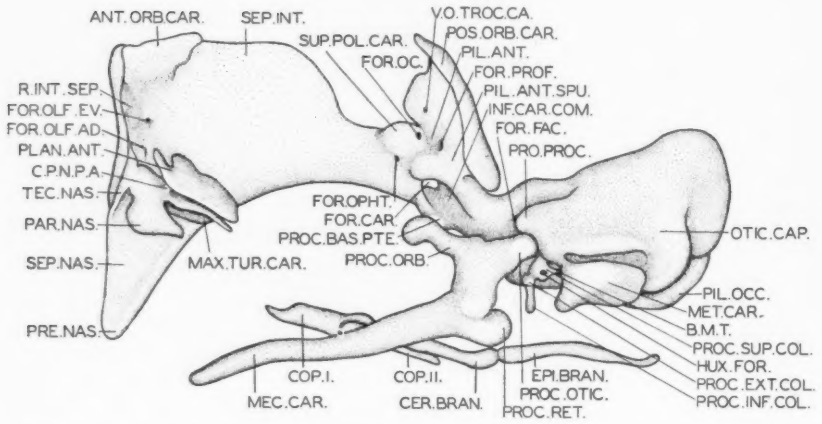

Fig. 13 (× = 16.4). Stage 5. Reconstruction of chondrocranium (norma lateralis).

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mesenchymatous tissue connecting these two structures. Copulae 1 and 2 have fused completely.

On the line of fusion the copula bears a cupshaped concavity on either side into which the medial tips of the ceratobranchials fit. The processus lingualis has chondrified in continuity with the anterior end of the fused copulae.

E STAGE V

Total length of embryo: 32.8 mm.

1 *The Basal Plate and Otic Capsules*

Only a few minor developments have taken place in this part of the skull so that on the whole it does not differ very much from the previous stage.

The basal plate shows the same degree of resorption in the vicinity of the chorda as in the previous stage. Only three hypoglossal foramina are present on each side. An important advancement has been made in the broadening and chondrification of the pila antotica spuria. As can be seen in the lateral reconstruction of this stage (Fig. 13), the latter is now a broad commissure between the posterior part of the orbital cartilage and the basal plate, situated lateral to the foramen for the profundus nerve.

The otic process of the pars quadrata now presses against the otic capsule (Fig. 13), and a cupshaped cavity on the anterior surface of the prominence of the lateral semicircular canal is the first indication of a future diarthrosis between the otic process and the auditory capsule. The prootic process has become longer.

2 *The Anterior End of the Chondrocranium*

The ethmoidal region has become more elongated. A new structure, the atrioturbinal cartilage has appeared in this region as a procartilaginous ridge projecting ventrally from the tectum nasi. It cannot be seen in the reconstruction as it is covered by the side wall of the capsule. In front of the planum antorbitale the maxilloturbinal cartilage can be seen, its posterior tip reaching as far back as the ventral edge of the planum antorbitale (Fig. 13).

Of special interest it the relation of the anterior part of the orbital cartilage to the interorbital septum. In this respect this stage is intermediate between the previous and the following stages. Between the anterior parts of the orbital cartilages the dorsal edge of the interorbital septum has grown upwards so that it is now more or less level with the dorsal edges of the orbital cartilages. (Compare figs. 12 and 13). The free dorsal portion of the orbital cartilage has rotated to a more vertical position, and its base is fused to the interorbital septum for a greater distance. Resorption of cartilage at the ventral edge of the anterior part of the orbital cartilage has taken place, exposing the olfactory nerve in this region and shifting the foramen olfactorium evehens postero-dorsally. The distance between the foramen olfactorium advehens and evehens is thus greater than in the previous stage. (Compare figs. 12 and 13).

3 *The Visceral Arches*

Few changes have taken place in the visceral arch skeleton. The otic process of the pars quadrata now abuts against the otic capsule. The pr. orbitalis has elongated considerably (Fig. 13). In the columella auris the pr. infracolumellaris has elongated. In the tissue filling Huxley's foramen the first indications of a bloodvessel are present. The pr. supracolumellaris has increased in length by extension of chondrification into the syndesmotic commissure connecting it to the ventral surface of the metotic cartilage (Fig. 13). The copula and first branchial arch are practically the same as in the previous stage except that more intercellular substance has been deposited in the tissue connecting the epibranchial and the ceratobranchial.

F STAGE VI

Total length of embryos: 33.6 mm. and 34.0 mm.

1 *The Basal Plate and Otic Capsules*

The acrochordal has been resorbed except for a thin ventral strip separating the hypophysial pit from the fenestra basicranialis posterior (Fig. 14). The oculomotor nerve, embedded in this structure in previous stages, has thus been

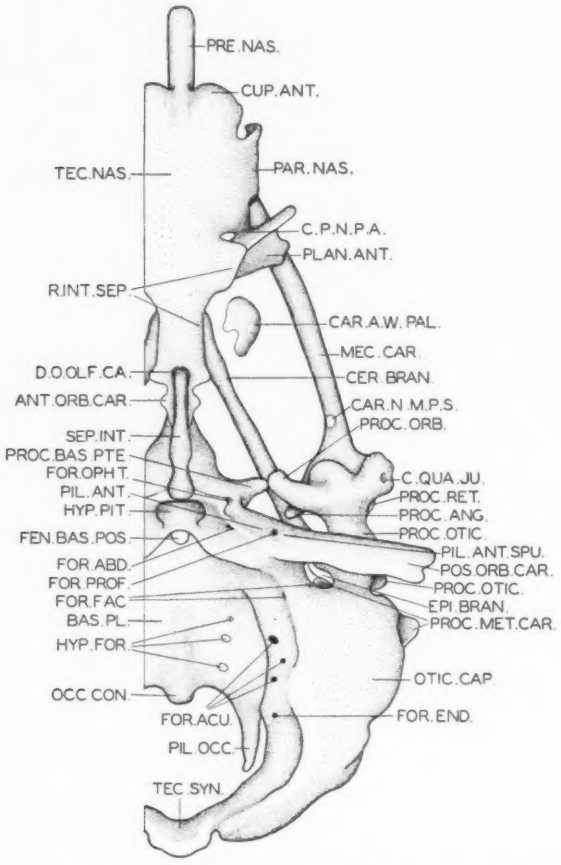

Fig. 14 (× = 16.6). Stage 6. (33.6 mm. embryo). Reconstruction of chondrocranium (norma dorsalis).

liberated. Resorption has also affected the medial part of the pila antotica so that is is now represented by a thin strip of cartilage only, lying medial to the foramen for the profundus nerve (Fig. 15). A thin membrane stretching between the pilae antoticae occupies the position of the medial portions of these structures and the dorsal part of the acrochordal. The anterior part of the chorda is embedded in this membrane. Behind the fenestra basicranialis posterior, except in the occipital condyle, the chorda no longer lies in a groove in the basal plate but more or less on the dorsal surface (Fig. 16). The pila occipitalis has increased in height considerably (Fig. 14).

All the walls of the otic capsule are now chondrified. On the medial surface of the canalicular part the wide foramen endolymphaticum has narrowed to a small foramen (Fig. 14). The lateral edge of the metotic cartilage and the ante-

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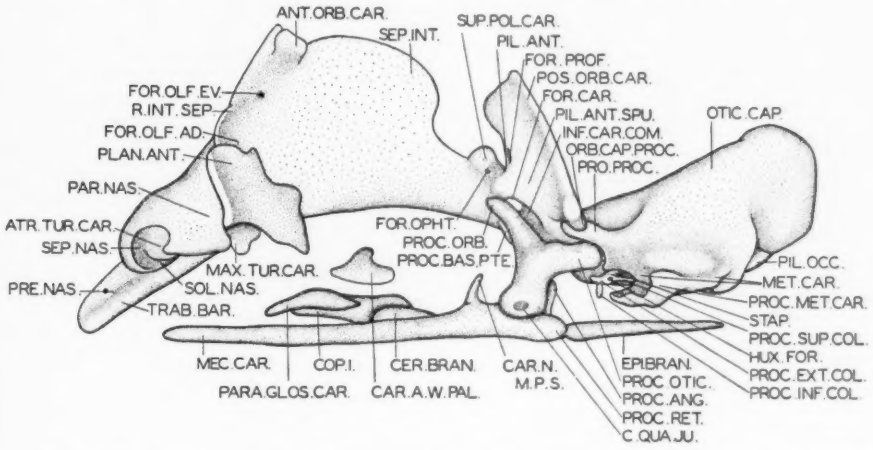

Fig. 15 (× = 13.2). Stage 6. (34 mm. embryo). Reconstruction of chondrocranium (norma lateralis).

rior edge lateral to the point where it has fused to the otic capsule in the previous stages have now fused to the prominentia semicircularis lateralis (Fig. 15). The anterior edge of the metotic cartilage forms the ventral part of the facet into which the lateral head of the otic process fits. An anteriorly directed plate-like outgrowth of the metotic cartilage lies ventro-lateral to the middle ear (Fig. 15). More posteriorly it forms a blind ending cavity between its medial surface and the ventral surface of the metotic cartilage. On the right side (Fig. 16) the anterior part of this process can be seen beneath the lateral part of the middle ear cavity and the posterior part of the meatus acusticus externus. Medial to this process the metotic cartilage itself underlies the medial part of the middle ear cavity and the recessus scalae tympani. On the left side (Fig. 16) the plane of section is farther back; the metotic cartilage here lies

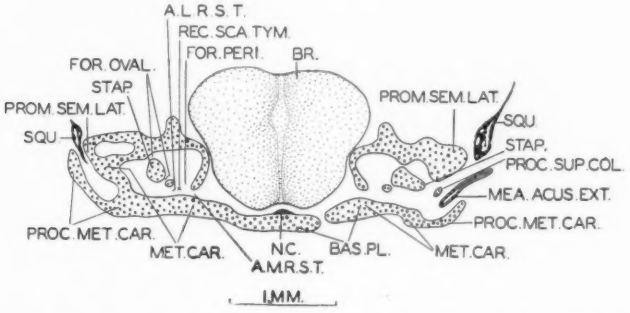

Fig. 16. Stage 6. (33.6 mm. embryo). Transverse section through the basal plate and otic capsules.

lateral and ventral to the cavity of the middle ear. Its lateral edge is fused to the prominentia semicircularis lateralis. The process of the ventral surface of the metotic cartilage projects upwards lateral to the rest of the cartilage. The cavity thus formed is closed dorsally by the ventral edge of the squamosal wedged in between the prominentia semicircularis lateralis and the dorsal edge of the process of the metotic cartilage. The lateral part of the posterior edge of the metotic cartilage is free from the otic capsule but the more medial part is syndesmotically connected to the prominence of the posterior semicircular canal.

2 *The Orbital Region*

Resorption of the anterior part of the orbital cartilage has been very rapid. Although the two embryos (Figs. 14 and 15) are identical as far as the rest of the chondrocranium is concerned, regression of the anterior part of the orbital cartilage has proceeded much further in the larger embryo, and the smaller one (33.6 mm.) may well be regarded as intermediate between the previous stage (32.8 mm.) and the embryo of 34.0 mm. as far as this aspect of the development is concerned. A description of the 33.6 mm. embryo will therefore be given first.

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No further upgrowth of the dorsal edge of the interorbital septum has occurred, and the whole of the posterior edge of the anterior part of the orbital cartilage has fused to the septum. In this way a trough has been formed behind the dorsal apertures of the olfactory canals (Fig. 14). This trough lodges the olfactory nerves and is bordered ventrally by the dorsal edge of the interorbital septum and laterally by the free dorsal portions of the anterior parts of the orbital cartilages (Fig. 17 a). Figs. 17 (a) and 17 (b) illustrate the relations of the anterior parts of the orbital cartilages to the interorbital septum. In a section (Fig. 17, a) through the region of the olfactory canal it can be seen that the canal is bordered medially, dorsally and ventrally by the interorbital septum and laterally by the anterior portion of the orbital cartilage.

The histological differences between the cartilage of the septum and the orbital cartilages are more pronounced than in the previous stages. Fig. 17 (b) shows a cross-section in the region behind the olfactory canals. The free dorsal portions of the anterior parts of the orbital cartilages lie almost vertical thus forming a deep trough between them.

In the older embryo regression of the anterior part of the orbital cartilage has proceeded further, and only a small piece remains cemented against the interorbital septum (Fig. 15). Through more resorption of the lateral wall of the olfactory canal the foramen olfactorium evehens has shifted to a more dorsal position. The posterior orbital cartilage has been subject to resorption along its dorsal edge. As a result of this the trochlear nerve has been liberated and now runs anteriorly above the medio-dorsal edge of this structure.

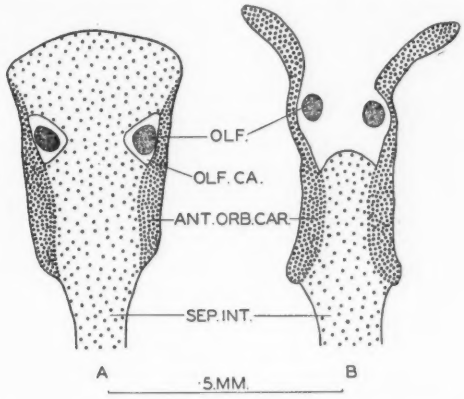

Fig. 17. Stage 6. (33.6 mm. embryo). (a) Transverse section through the dorsal part of the interorbital septum in the region of the olfactory canals. (b) Transverse section through the dorsal part of the interorbital septum in the region of the trough lodging the olfactory nerves.

Progressive lateral fusion between the ventral edge of the posterior part of the orbital cartilage and the basal plate has increased the breadth of the pila antotica spuria (Fig. 15). The postero-ventral edge has developed a posteriorly directed orbitocapsular process, the tip of which lies lateral to the prootic process of the canalicular part of the otic capsule (Fig. 15).

3 *The Nasal Capsules*

The nasal capsules have developed very rapidly in the meantime. In the previous stage (32.8 mm.) the roof, side walls and turbinal cartilages had not developed much further than in the four embryos ranging from 29.6 to 31.2 mm. in length. In the two embryos used in the description of this stage all these structures are well developed.

A roof and side wall for each capsule have been formed by the overgrowing parietotectal (Figs. 14, 15 and 18). A cupola anterior clothes the anterior end of the nasal sac and sends back a small process beneath the latter (Figs. 15 and 18). This process forms a small floor to the anterior part of the vestibule, and on one side its medial edge is fused to the lateral surface of the septum nasi for a short distance. At its lowest point the floor does not extend ventrally below the level of the dorsal edge of the trabecular bar. Attention will be called to this point later. The ridge-like procartilaginous anlage of the atrioturbinal cartilage has chondrified as a large T-shaped structure consisting of a medioventrally directed primary lamella bearing medially and laterally directed secondary lamellae (Figs. 18 and 19), except for an anterior part projecting into the fenestra narina (Fig. 15). The latter is a rounded notch bordered anteri-

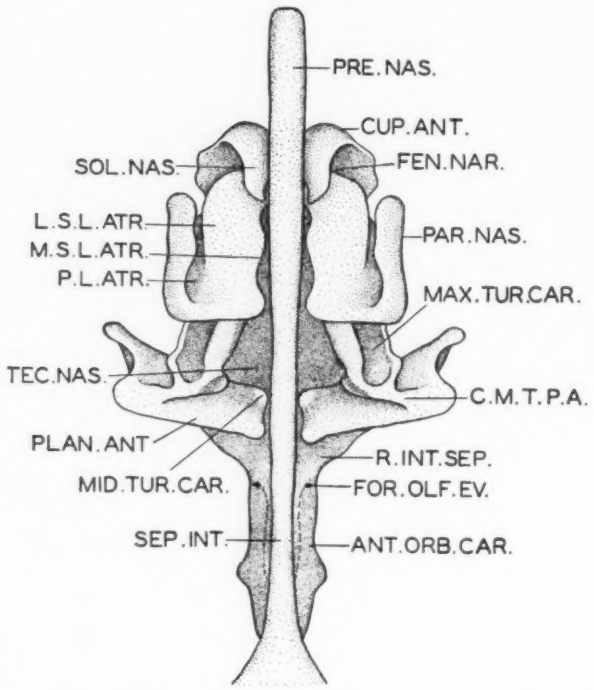

Fig. 18 (× = 25.2). Stage 6. (33.6 mm. embryo). Reconstruction of nasal capsules (norma ventralis).

orly by the cupola anterior, antero-ventrally by the posteriorly directed process of the cupola and posteriorly by the anterior edge of the paries nasi.

Medio-ventrally a large gap exists between the antero-ventral corner of the paries nasi and the process of the cupola anterior (Fig. 18). The posterior edge of the atrioturbinal cartilage is continued uninterruptedly into the posterior edge of that part of the paries nasi covering it laterally. The common edge of these two structures forms a ventrally directed transverse ridge (Fig. 18). Behind the atrioturbinal cartilage the paries nasi does not extend as far ventrally as in the region of the turbinal so that the posterior part of the scroll-like maxilloturbinal cartilage is uncovered laterally. Its anterior part is situated medio-dorsal to the posterior part of the atrioturbinal. Its posterior end is fused to the anterior surface of the planum antorbitale near its ventro-lateral corner (Fig. 18).

The latter structures has increased in size especially along the dorsal part of its medial edge carrying the ventral border of the foramen olfactorium advehens postero-dorsally, so that the latter thus becomes a narrow slitlike opening. The course of the olfactory nerve and the profundus branch of the trigeminal

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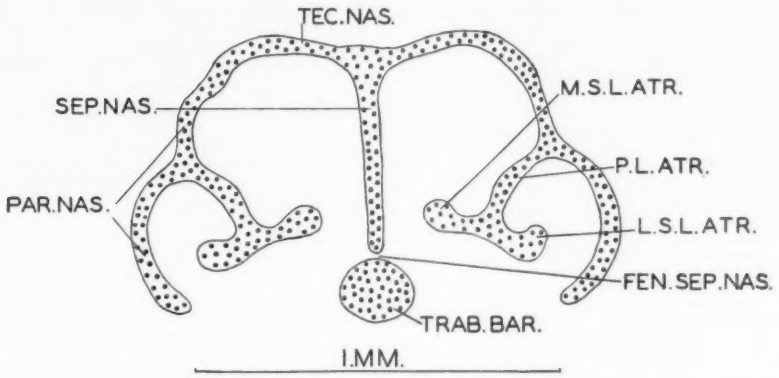

Fig. 19. Stage 6. (33.6 mm. embryo). Transverse section through the nasal capsules in the region of the fenestra septi nasi.

l.c. nerve is as follows: both nerves pass anteriorly over the dorsal edge of the planum antorbitale. Above the foramen olfactorium advehens the profundus divides into lateral and medial branches. The former is continued antero-laterally in the groove between the anterior surface of the dorsal part of the planum antorbitale, and the side wall of the nasal capsule. The medial branch accompanies the olfactory nerve downwards through the foramen olfactorium advehens, closely attached to the internasal septum immediately behind the craniofacial fenestra. It is continued forwards dorso-lateral to the trabecular bar for some distance but then bends downwards to be continued anteriorly below the latter. On the anterior surface of the medio-ventral corner of the planum antorbitale an anteriorly directed vertical ridge has developed. PARKER (1876) described an irregular ridge on the anterior surface of the planum antorbitale in *Falco tinnuculus* as a "middle turbinal". Whether this ridge in *Pyromelana* is the same as PARKER's "middle turbinal" is difficult to say as PARKER did not describe the exact position of the structure. Posteriorly the medio-ventral corner of the planum antorbitale has developed a postero-medially directed process (Fig. 15).

In the septum nasi a fenestra septalis and a craniofacial fenestra have developed through resorption of the septal cartilage. The former is a long narrow slit in the anterior region of the septum nasi, situated immediately above the trabecular bar (Fig. 19). The craniofacial fenestra is situated farther posteriorly, a short distance anterior to the posterior ends of the maxilloturbinal cartilages. It is more or less oval in shape and closed by a membrane which represents the perichondria of the resorbed septum nasi.

The part of the septum nasi above the trabecula bar is very thin in comparison with the bar itself (Fig. 19) except for the posterior region of the capsules where the dorsal part is wedge-shaped. The trabecular bar can easily be

distinguished from the rest of the septum nasi, as far back as the posterior tip of the planum antorbitale. It attains its greatest diameter in the anterior region of the nasal capsules and in the prenasal process. In contrast to the rest of the septum nasi it possesses a well-developed perichondrium, and its chondrocytes are highly hypertrophied. The perichondrium covers the ventral, lateral and dorso-lateral surfaces of the trabecular bar. The transition between the perichondrium of the trabecular bar and the relatively undifferentiated perichondrium of the more dorsal part of the septum is very sharp. In the region of the fenestrae a wall-developed perichondrium surrounds the whole trabecular bar.

The hypertrophied condition of the chondrocytes is found almost to the tip of the prenasal process, which latter can be divided into two regions: a longer posterior consisting of the trabecular bar bearing a dorsal crest and a shorter anterior part consisting of the trabecular bar only. The crest of the posterior part of the prenasal process is continued posteriorly into the septum nasi, and it shows the same degree of development as the latter.

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4 *The Visceral Arches*

The synchondrotic continuity between the ventral end of the pars quadrata and Meckel's cartilage has broken down. A synovial cavity will develop later but at this stage the space between the two structures is filled with connective tissue. On the lateral surface of the lower part of the pars quadrata a concavity lodging the posterior end of the quadratojugal has developed (Fig. 15). A cartilaginous protuberance has appeared on the medial surface of the lower part of the pars quadrata. Later a synovial cavity will develop between this protuberance and the posterior end of the pterygoid. The process develops as a chondrification in the posterior end of the same mesenchymatous tract in which the pterygoid ossifies, and in this stage it is separated from the posterior tip of the developing pterygoid by a zone of mesenchymatous tissue.

The distal end of the medial portion of the hyoidean visceral cleft has enlarged to form a middle ear cavity into which the columella auris projects. A tympanic membrane has been formed between the lateral wall of the middle ear and the medial wall of the external ear passage. The processus supracolumellaris has elongated postero-dorsally by deposition of more intercellular substance in the syndesmotic connexion between its tip and the ventral surface of the metotic cartilage.

Lateral to the anterior part of the copula two new elements, the paraglossal cartilages, have chondrified (Fig. 20). Their mesenchymatous anlagen were already present in the two previous stages. Their anterior portions are more or less parallel to the median copula, while their posterior portions diverge laterodorsally. On the boundary between these two portions the medial edges of the paraglossals approach each other very closely above the anterior part of the

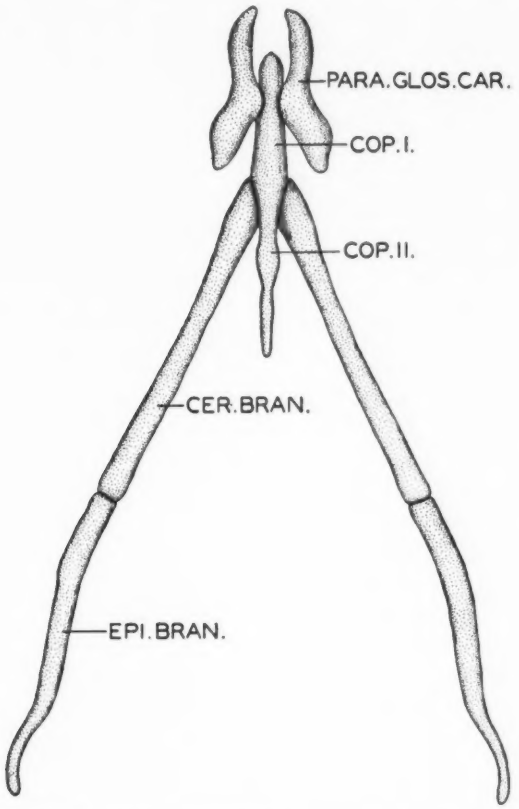

Fig. 20 (× = 30.7). Stage 6. (33.6 mm. embryo). Reconstruction of hyoid apparatus (norma ventralis).

copula. In this region a stout syndesmotic connexion is present between their medial edges. It is still possible to determine the line of demarcation between the epibranchial and the ceratobranchial. In both, the chondrocytes are highly hypertrophied, and the middle portion of the ceratobranchial is surrounded by a thin layer of perichondral bone.

5 *Other Cartilaginous Structures*

Two other cartilaginous structures which cannot be considered as part of the chondrocranium have now appeared. A cartilaginous nodule has chondrified in the tendon of the musculus pseudotemporalis superficialis. It is closely applied to the dorsal surface of Meckel's cartilage a short distance in front of its articulation with the pars quadrata (Figs. 14 and 15). Posteriorly, continuity of matrix exists between this nodule and Meckel's cartilage, but more anteriorly

a thin membrane is discernible between them. In a slightly younger embryo it can be seen that the nodule arises as a separate centre of chondrification in the band of connective tissue stretching between the ventral end of the m. pseudotemporalis superficialis and the dorsal edge of Meckel's cartilage.

In the palate a triangular piece of cartilage has appeared along the lateral edge of the posterior part of the palatine (Figs. 14 and 15). It is hyaline with a well differentiated perichondrium. Its medial edge is closely applied to the lateral edge of the palatine so that its perichondrium passes over uninterruptedly into the periosteum of the latter. W. K. PARKER (1876) remarked that the elbowed form of the palatines near their hinder third is a characteristic of the *Passerine* palate, and in the embryo of the Brown Linnet *(Linota cannabina)* he observed that "this outbent part does not ossify by 'first intention', but lingers on until it is solid hyaline cartilage, and then receives its own endosteal core" (p. 109). Parker considered it to be an "arrested" transverse element, and referred to it as the transpalatine in the numerous forms in which he described this structure. It is most improbable that it is homologous with a transpalatine or os transversum such as is present in reptiles, and therefore this structure will be referred to as the cartilage associated with the palatine. Examination of early stages showed that it chondrifies in the same mesenchymatous anlage in which the palatine ossifies. A more detailed account of its earlier development will be given when its possible homologies are discussed at the end of this paper.

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G STAGE VII

Total length of embryo: 35.2 mm.

1 *The Basal Plate and Otic Capsules*

No important structural changes have taken place in the basal plate. Hypertrophied chondrocytes in the vicinity of the chorda, in the pila occipitalis and pars lateralis of the occipital region are the first indications of future ossification in these regions. The chondrocytes in the medial part of the metotic cartilage are also hypertrophied. The lateral part of the posterior edge of the metotic cartilage has fused to the postero-lateral edges of the prominenta semicircularis lateralis leaving, however a small foramen (Fig. 21) through which bloodvessels enter the cavum metoticum. More medially the posterior edge of the metotic cartilage is fused for some distance to the prominentia semicircularis posterior. In the region of the prominentia semicircularis superior the posterior edge of the metotic cartilage and more medially the anterior edge of the pila occipitalis are syndesmotically connected to a cartilaginous ridge projecting ventrally from the prominentia semicircularis superior. This ridge has chondrified in the thin procartilaginous tissue connecting the posterior edge of the

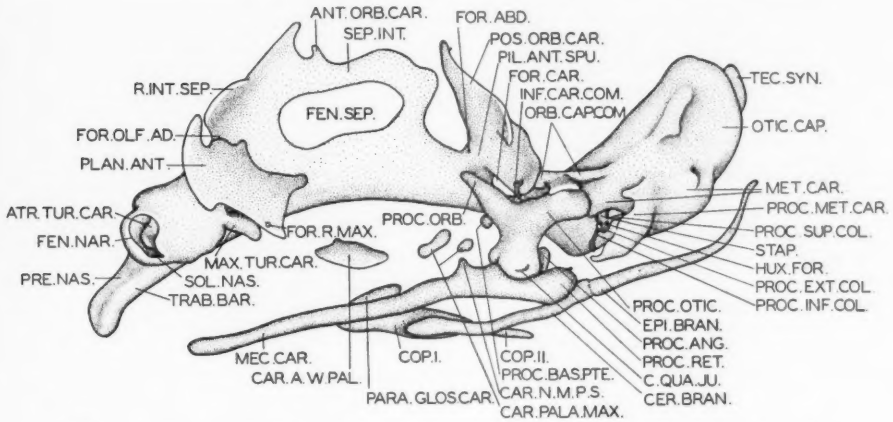

Fig. 21 ($\times = 10.9$). Stage 7. Reconstruction of chondrocranium (norma lateralis).

metotic cartilage to the otic capsule in previous stages. The dorsal tip of the pila occiptialis is applied very closely to the ventral side of the prominentia semicircularis superior, but a common perichondrium is still discernible between the two structures. A low ridge has developed along the lateral part of the roof of the otic capsule (Fig. 21).

2 *The Orbital Region*

A large fenestra septalis has developed in the interorbital septum by resorption of cartilage (Fig. 21). Along its dorsal edge signs of absorption can also be detected. The anterior part of the orbital cartilage has been completely resorbed on the one side. A very small remnant is present on the other side and attached to the dorsal edge of the interorbital septum (Fig. 21); it is histologically easily distinguishable from the cartilage of the septum. The whole lateral wall of the olfactory canal has thus disappeared, and the olfactory nerve traverses the orbit along its entire length. Resorption has also taken place along the dorsal edge of the suprapolar cartilage and the lateral part of the dorsal edge of the posterior part of the orbital cartilage. The orbitocapsular process of the posterior part of the orbital cartilage has fused to the lateral surface of the anterior part of the prootic process of the otic capsule, thus converting the incisura antotica spuria into a foramen antoticum spurium for the maxillary and mandibular branches of the trigeminal nerve. Fused to the lateral edge of the orbitocapsular portion of this commissure, between the posterior part of the orbital cartilage and the otic capsule, is a cup-shaped cartilaginous structure (Fig. 21) lining the facet on the ventral edge of the squamosal with which the lateral head of the pr. oticus of the pars quadrata articulates. This plate of

cartilage has chondrified in continuity with the orbitocapsular process in the mesenchymatous tissue between the developing lateral head of the otic process and the developing squamosal. T. J. PARKER (1891) described this structure in the Kiwi and he considered it possible as a neomorphic structure and comparable to and possible homologous with the meniscus of mammals.

3 *The Nasal Capsules*

The trabecular bar in the ethmoidal region has reached its maximal development in this stage; in subsequent stages it will be resorbed very rapidly. The most important advances made in the nasal capsule are the formation of a zona annularis behind the fenestra narina and the chondrification of an accessory atrioturbinal cartilage. The posteriorly directed process of the cupola anterior has fused to the antero-ventral corner of the paries nasi, delimiting the fenestra narina postero-ventrally. Fusion between the medial edge of this process and the lateral surface of the septum nasi, some distance above the trabecular bar, has completed the zona annularis ventrally. The floor thus formed beneath each vestibulum bears a short posteriorly directed process (Fig. 22).

A ridge-like structure has chondrified along the roof of the capsule and along the lateral surface of the septum nasi, just behind the fenestra narina. A similar structure was described by SUSCHKIN (1899) in *Tinnunculus* as the "accessorische Vorhofsmuschel". DE BEER and BARRINGTON (1934) in order to establish conformity in the nomenclature of the turbinal cartilages introduced the name atrioturbinal for the structure described by SUSCHKIN as the "Vorhofsmuschel". In this paper the name accessory atrioturbinal cartilage has been coined for the extra turbinal in the anterior region of the nasal capsule. Fig. 22 is a reconstruction of the ethmoidal region in which the side wall, the atrioturbinal cartilage, the maxilloturbinal cartilage, and most of the roof and planum antorbitale have been omitted to show the position of the accessory atrioturbinal. It begins as a low ridge on the ventral surface of the medial part of the tectum nasi and is continued ventrally along the lateral surface of the septum and terminates a short distance above the fenestra septi nasi. The common posterior end of the atrioturbinal and paries nasi has developed a medial extension, the ventral edge of which lies below the level of the trabecular bar. Posteriorly the medial extension bears a short backwardly directed process lying along the lateral edge of the azygous vomer. The ventro-lateral corner of the planum antorbitale has extended laterally beneath the eye-ball enclosing the maxillary branch of the trigeminal nerve in a foramen (Fig. 21). The dorsal corner of the medial edge has grown out into a short posteriorly directed process.

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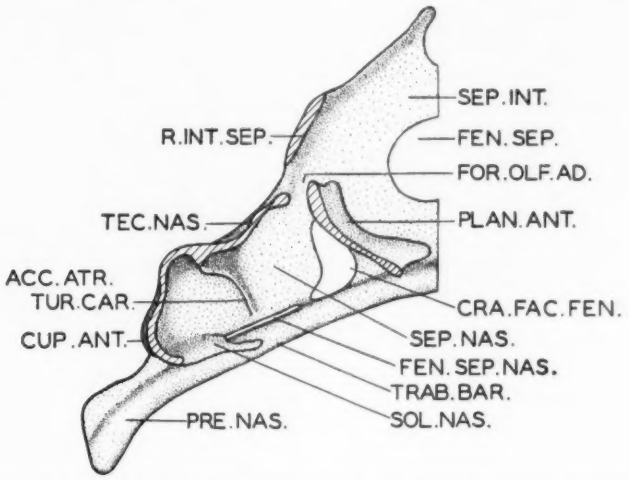

Fig. 22 (× = 16.6). Stage 7. Reconstruction of nasal capsule (norma lateralis) in which the side wall and the atrioturbal and maxilloturbinal cartilages have been removed.

4 *The Visceral Arches*

The pars quadrata has now assumed the typical adromoeognathic form by the development of a lateral head of the pr. oticus articulating with the ventral edge of the squamosal. A synovial cavity has already been formed between the lateral head of the otic process and the cartilage lining the cupshaped surface on the ventral edge of the squamosal. Synovial cavities have also developed between the articulating surfaces of Meckel's cartilage and the ventral end of the pars quadrata, between the medial head of the otic process and between the process on the medial surface of the pars quadrata and the posterior end of the pterygoid. Faint traces of intercellular deposit between the cup-shaped posterior end of the pterygoid and the synovial cavity indicate the future formation of a cartilaginous lining for the concavity of the pterygoid. The paraglossal cartilages have elongated considerably.

5 *Other Cartilaginous Structures*

The cartilaginous nodule in the tendon of the m. pseudotemporalis superficialis has completely fused to the dorsal surface of Meckel's cartilage (Fig. 21). Antero-lateral to it two cartilagines palatomaxillares have chondrified in the tendons of the m. adductor mandibulae externus. Ventrally these cartilages are connected to the undifferentiated tissue around the dorsal edge of the developing supra-angular. (The name cartilagines palatomaxillares has been introduced in this paper for reasons that will be explained later.)

H STAGE VIII

Total length of nestling: 37.0 mm.

The Basal Plate and Otic Capsules

Perichondral ossification has set in in the basal plate. Behind the fenestra basicranialis posterior a thin layer of perichondral bone is present on the cerebral and ventral surfaces of the region of hypertrophied cartilage in the vicinity of the chorda. Perichondral bone is also present on both surfaces of the pars lateralis of the occipital region. The pila occipitalis is clothed by a layer of perichondral bone except for its dorsal tip which is synchondrotically connected to the otic capsule (Fig. 23). Three hypoglossal foramina, of which the posterior one is the largest, are present on each side. This appears to be the final (adult) number in *Pyromelana,* because the same number was present in the two previous stages and in several nestlings older than the one used for the description of the present stage.

The posterior edge of the metotic cartilage is fused to the otic capsule along its entire length. The anteriorly directed plate-like outgrowth of the ventral surface of the metotic cartilage has enlarged, so that it now covers the greater part of the middle ear cavity ventro-laterally (Fig. 23).

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2 The Orbital Region

The fenestra septalis has increased in size; a remnant of the anterior part of the orbital cartilage still lingers on as a thin strip (·015 mm. in length) along the dorsal edge of the septum. It represents part of the side wall of the trough lodging the olfactory nerves in previous stages. In older nestlings all traces of the anterior part of the orbital cartilage have completely disappeared. The posterior part of the orbital cartilage and the pila antotica spuria, have undergone no further development. No trace of future ossification is yet present in these structures.

The anterior part of the ventral edge of the interorbital septum is still free from the underlying rostrum, but for some distance anterior to the fenestra hypophyseos the latter has begun to invade the ventral septal edge, and a layer of perichondral bone has started to spread upwards along the lateral surface of the ventral part of the interorbital septum. In the region of the hypophysial pit the chondrocytes are highly hypertrophied, and the greater part of this is covered by a layer of perichondral bone. The suprapolar cartilage has been almost completely resorbed except for a small part on the dorsal edge of the hypophysial pit (Fig. 24). A thick layer of perichondral bone surrounds the pr. basipterygoideus, and along its dorsal surface periosteal buds indicate the beginning of enchondral ossification. Although the pterygoid approaches the

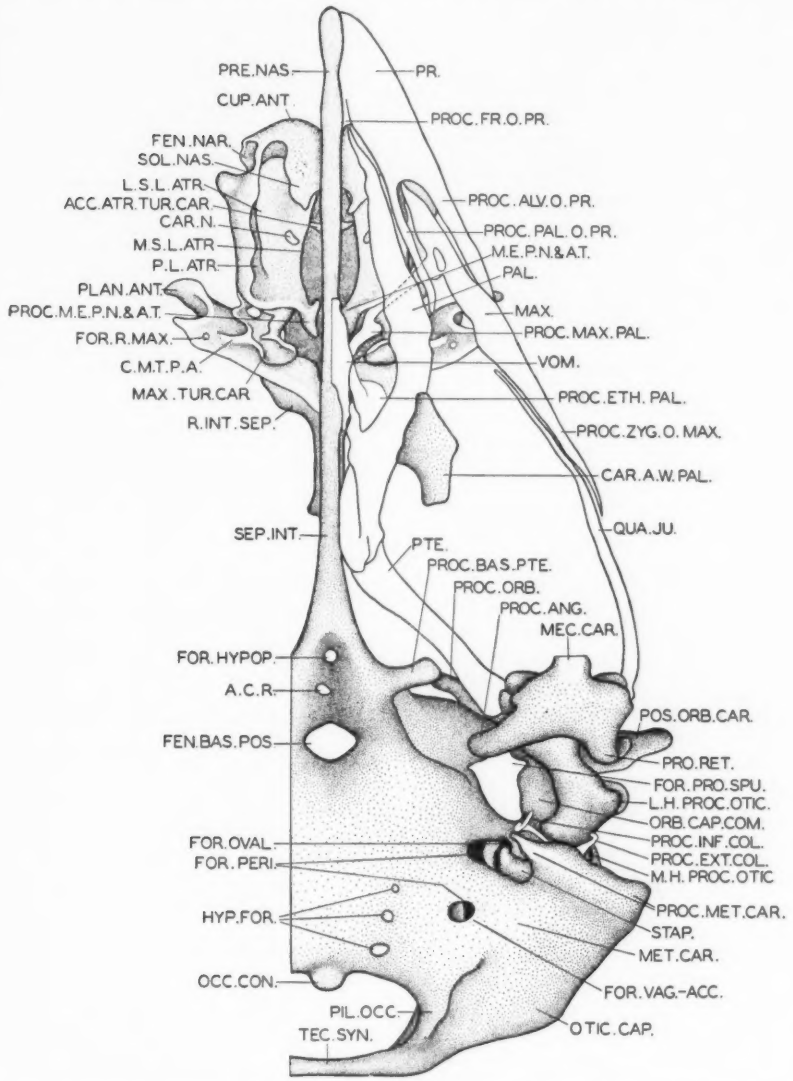

Fig. 23 ($\times = 11.7$). Stage 8. Reconstruction of chondrocranium and of membrane bones of the palate (norma ventralis).

tip of the basipterygoid process quite closely at this stage (Fig. 23) and the space between them is devoid of any tissue, there is no synovial cavity between them. In the process of ossification and pneumatization the basipterygoid process becomes reduced. In an old nestling a remnant of it can still be made out but in the adult it has completely disappeared.

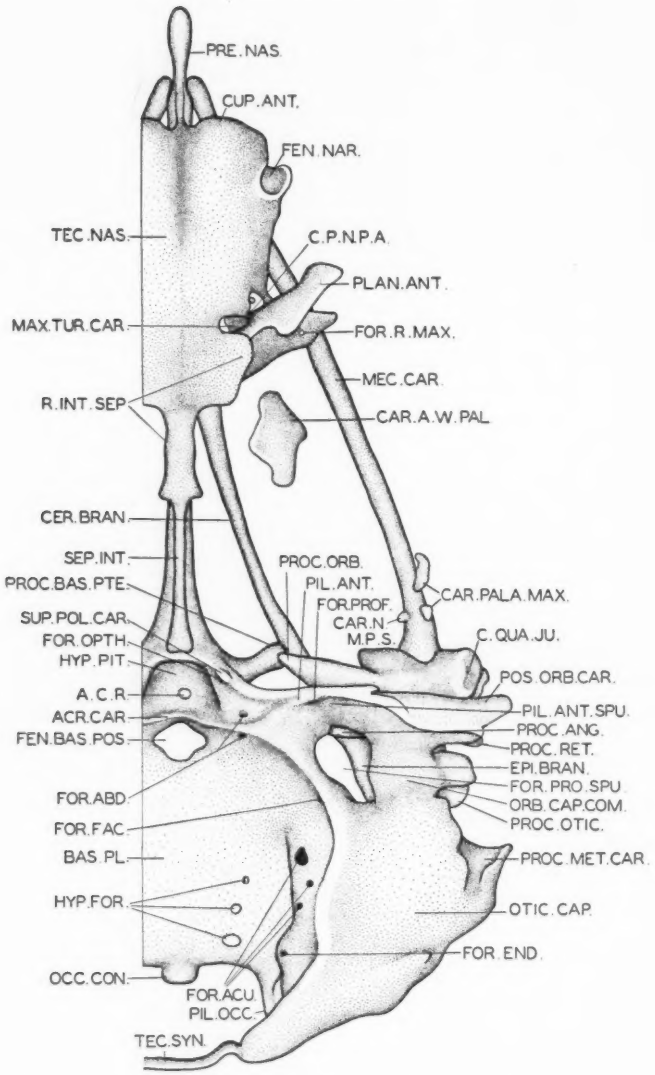

Fig. 24 ($\times = 11.7$). Stage 8. Reconstruction of chondrocranium (norma dorsalis).

The parasphenoid ossification has spread dorsally through the fenestra hypophyseos to form an osseous plate covering the anterior part of the floor of the hypophysial pit. A narrow cavity is now present between this part of the parasphenoid and the layer of perichondral bone on the dorsal surface of the cartilaginous floor of the hypophysial pit. Resorption of the floor of the hypophysial pit allows of greater penetration of the parasphenoid ossification

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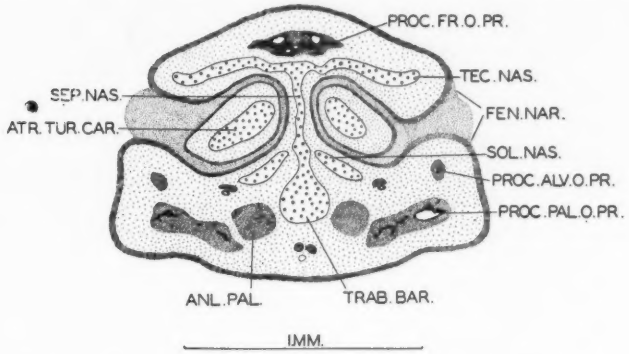

Fig. 25 (a). Stage 6. Transverse section through the nasal capsules in the region of the fenestra narina.

into the pit. In this stage another aperture has been formed between the fenestra hypophyseos and the fenestra basicranialis posterior (Figs. 23 and 24).

3 *The Nasal Capsules*

Although the nasal capsules have not yet completed their development in some areas, regression has set in in others. It is most noticeable in the prenasal process and in the part of the nasal septum ventral to the vestibular floor. Anteriorly the prenasal process still has the trabecular bar as well as its crest unaffected by the process of resorption, but more posteriorly both the crest and trabecular bar have thinned down considerably (Fig. 23). Just anterior to the cupolae anteriores the crest becomes separated from the trabecular bar and this discontinuity between the trabecular bar and the septum is continued backwards as far as a point anterior to the posterior edge of the vestibular floor. Except for a small area beneath the cupolae anteriores, the part of the septum between the trabecular bar and the vestibular floor has been resorbed, and a thin strip of connective tissue indicates its previous position. Figs 25 (a) and (b) showing transverse sections through this region of the 6th and present stages clearly illustrate the changes. The anterior part of the trabecular bar beneath the vestibular floor has not been subject to resorption, as can be seen in Fig. 25 (b). Towards the posterior end of the floor and as far back as a point just posterior to the craniofacial fenestra resorption is noticeable along the ventral edge of the trabecular bar. It is particularly conspicuous in that part of the trabecular bar bordering the craniofacial fenestra ventrally.

Approximately in the same transverse plane as the accessory atrioturbinal cartilages a cartilaginous nodule has appeared on each side and some distance away from the trabecular bar (Figs. 23 and 26). A strand of connective tissue stretches between each nodule and a procartilaginous protuberance on the lateral

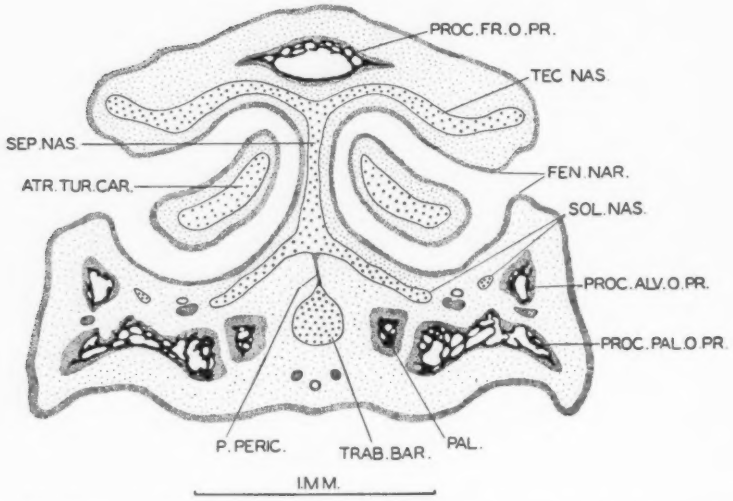

Fig. 25 (b). Stage 8. Transverse section through the nasal capsules in the region of the fenestra narina.

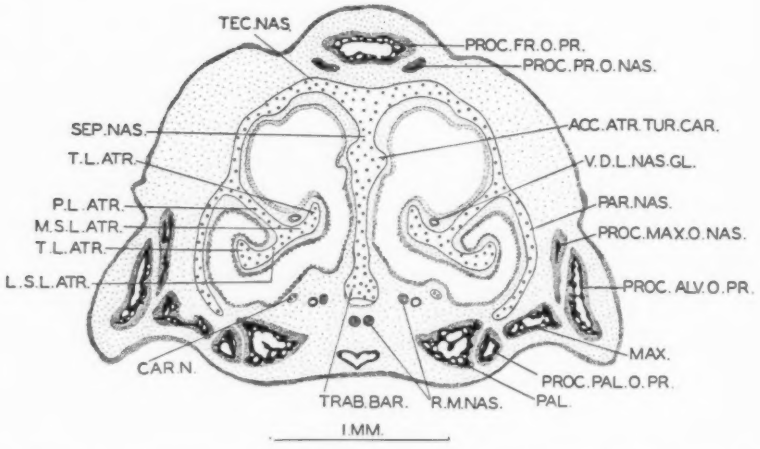

Fig. 26. Stage 8. Transverse section through the nasal capsules in the region of the cartilaginous nodules.

surface of the septum nasi above the trabecular bar. The medial extension of the common posterior end of the side wall and atrioturbinal cartilage is better chondrified than in the previous stage, and its posteriorly directed process has elongated (Fig. 23). Between the medial edges of the extensions of both sides the vomer is wedged in (Fig. 27). The medial edges rise upwards to form the lateral walls of a deep trough lodging the base of the nasal septum; the floor

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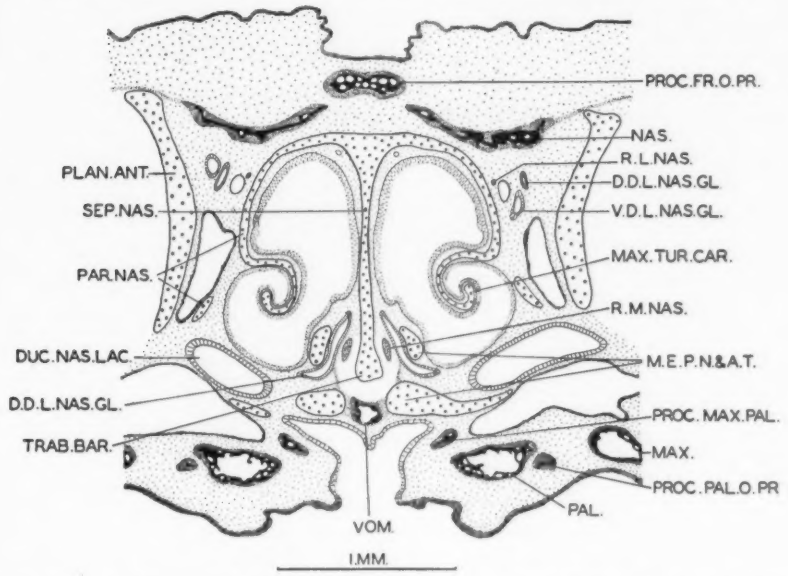

Fig. 27. Stage 8. Transverse section through the nasal capsules in the region of the medial extension of the paries nasi and the atrioturbinal cartilage.

of the trough is formed by the azygous vomer. The dorsal duct of the nasal gland enters this trough through a foramen in its lateral wall (Fig. 27).

The posteriorly directed process of the dorsal corner of the medial edge of the planum antorbitale is now closely applied to the interorbital septum.

The inferior oblique eye muscle attaches to this process as well as to the septum just below it. The posteriorly directed process of the ventral corner of the medial edge of the planum antorbitale has elongated. Although it is very closely applied to the lateral surface of the interorbital septum, there is no continuity of matrix, but a common perichondrium is discernible between them.

The originally solid anlagen of the lacrimonasal duct and ducts of the lateral nasal gland have each developed a lumen. The former opens posteriorly into the antero-lateral corner of the orbit by means of two orifices, a dorsal and a ventral. It is continued antero-medially below the edge of the planum antorbitale to open into the choana of its side immediately behind the medial extension of the common posterior edge of the paries nasi and the atrioturbinal cartilage (Fig. 27). The nasal gland stretches from the antero-dorsal to the postero-dorsal corner of the orbit. Its longer posterior portion consists of a single duct but in its anterior portion diverticula are present. The anterior part is situated lateral to the flange of the interorbital septum and the lateral edge of the anterior tip of the frontal. In *Anas* (GREWE, 1951) the anterior tip of the nasal gland is situated just behind the postero-dorsal end of the lacrimal. This bone is absent

in *Pyromelana*. From the anterior end of the gland two ducts, a small dorsal and a longer ventral are continued antero-medially over the dorsal edge of the planum antorbitale into a space bordered dorsally by the nasal, laterally by the anterior surface of the planum antorbitale and medially by the side wall of the nasal capsule (Fig. 27). Just behind the common posterior end of the atrioturbinal cartilage and side wall of the capsule, both ducts turn sharply downwards. The ventral duct enters the capsule beneath the ventral edge of the side wall and is continued anteriorly above the medially directed secondary lamella of the atrioturbinal cartilage (Fig. 26). Its opening into the vestibulum is situated a short distance in front of the anterior end of the maxilloturbinal cartilage. The course of the dorsal duct is more complicated. It leaves the company of the ventral duct where the latter enters the nasal capsule, is continued postero-ventrally and pierces the side wall of the trough lodging the base of the nasal septum. (Fig. 27). Immediately after its entrance into the trough it bends dorsally and is continued forwards along the septum nasi about halfway between its base and the roof of the capsule. It opens into the vestibulum a short distance behind the orifice of the ventral duct.

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4 *The Visceral Arches*

There have been no further changes in the pars quadrata and Meckel's cartilage. In a reconstruction it can be seen that Meckel's cartilage has lost the curvature present in the younger stages and is completely straight. No symphysis is formed between the anterior tips of these cartilages. The pr. angularis medialis is well developed and projects postero-dorsally from the posterior end of Meckel's cartilage. (Fig. 23.)

The columella auris is now fully developed. The only change that will take place in later stages is the ossification of the stapes. The pr. infracolumellaris is a long thin structure with its ventral tip bent outwards and embedded in the muscles. The pr. supracolumellaris has elongated posteriorly, its posterior portion lying in the cavity formed between the metotic cartilage and the upgrowing part of the process of its ventral surface. It is embedded in the tympanic membrane, and the syndesmotic connexion between its tip and the ventral surface of the metotic cartilage has broken down.

Between the medial edges of the paraglossal cartilages and the dorsal and dorso-lateral surfaces of the anterior part of the copula a large synovial cavity has developed in the region of the stout syndesmotic connexion between the medial edges of the paraglossal cartilages. Another synovial cavity has been formed between the medial tip of the ceratobranchial and a concavity on the lateral surface of the copula. The layer of perichondral bone around the ceratobranchial has increased in thickness, and here and there periosteal buds indicate the beginning of enchondral ossification.

The line of demarcation between the epibranchial and the ceratobranchial can still be made out although the connecting tissue already contains a large amount of intercellular deposit. In the adult, epibranchial and ceratobranchial will ossify as one continuous structure.

5 *Other Cartilaginous Structures*

The cartilage associated with the palatine has increased in size; its position in the palate is shown in Fig. 23. The cartilagines palatomaxillares are better differentiated than in the previous stage and they lie close against the dorsal edge of the supra-angular.

I STAGE IX

Total length of nestling: 39 mm.

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9
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Although ossification and pneumatization of the rest of the chondrocranium has set in, important changes are still taking place in the nasal capsules. In this and in the following stages the nasal capsules only will be described.

The Nasal Capsules

Resorption of the trabecular bar can now be observed along the whole length of the structure in the ethmoidal region; it is most conspicuous in the prenasal process and in the region underlying the fenestra septalis and the craniofacial fenestra. The prenasal process has been greatly affected by resorption. Although signs of resorption are present its anterior tip is in a better state of preservation than the part just anterior to the cupolae anteriores, where the stout process of previous stages has been reduced to thin interrupted dorsal and ventral struts. As in the previous stage the best preserved part of the trabecular bar is that underlying the anterior part of the solum nasi.

The nodules of cartilage described in the previous stage as lying more or less in the same transverse plane as the accessory atrioturbinal cartilage have not yet chondrified in this specimen. Condensations of tissue are present in corresponding positions. In the previous stage the trough lodging the ventral edge of the septum in the region of the craniofacial fenestra was bordered only ventrally by the vomer. The condition still applies to that part of the trough anterior to the foramina for the dorsal ducts of the lateral nasal glands, but immediately behind these foramina the vomer sends up a thin lamella along the medial edge of each of the medial extensions of the common posterior edges of the atrioturbinals and side walls of the capsules (Fig. 28).

Attention must now be drawn to a small nodule of cartilage (·075 mm. long) beneath the posterior part of the planum antorbitale. It is present only on

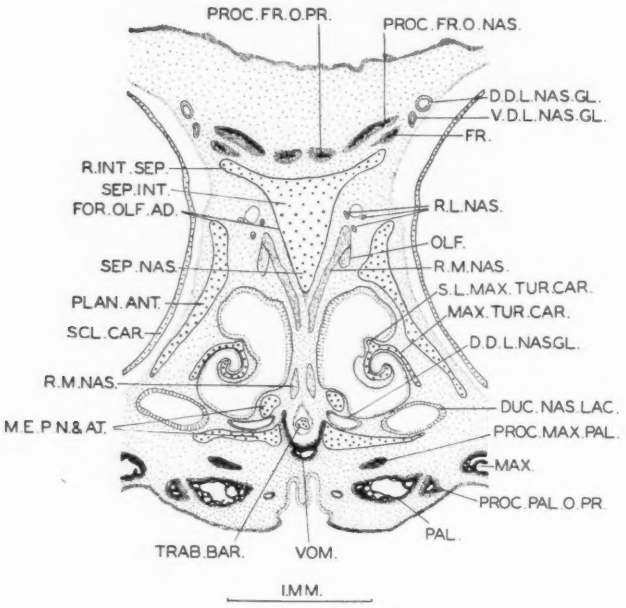

Fig. 28. Stage 9. Transverse section through the nasal capsules in the region of the medial extension of the paries nasi and the atrioturbinal cartilage.

the one side and is situated medio-dorsally to the anterior tip of the processus ethmopalatinus (Fig. 29).

PARKER (1876) introduced the name vomerine cartilages for cartilaginous structures occurring in this region of the avian palate.

Near the posterior end of the maxilloturbinal cartilage a small medio-dorsally directed secondary lamella has begun to develop on its dorsal surface (Fig. 28).

J STAGE X

Total length of nestling: 57 mm.

The Nasal Capsules

The trabecular bar has almost completely disappeared except for a thin strut of cartilage underlying the posterior part of the solum nasi (Fig. 30). Its anterior end lies well below the solum nasi to which it is connected by a strand of connective tissue, but posteriorly it is still connected to the dorsal part of the septum. The posterior part of this strut consists of a core of hyaline cartilage surrounded by a layer showing sign of resorption. Behind the strut the previous existence of the trabecular bar is still indicated by a strip of connective tissue containing small nodules of cartilage here and there, especially below the cranio-

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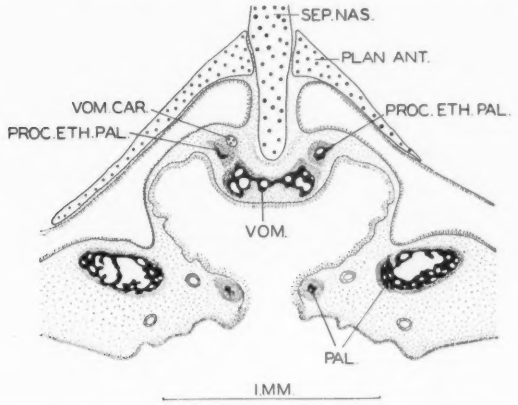

Fig. 29. Stage 9. Transverse section through the palate showing the position of the vomerine cartilage.

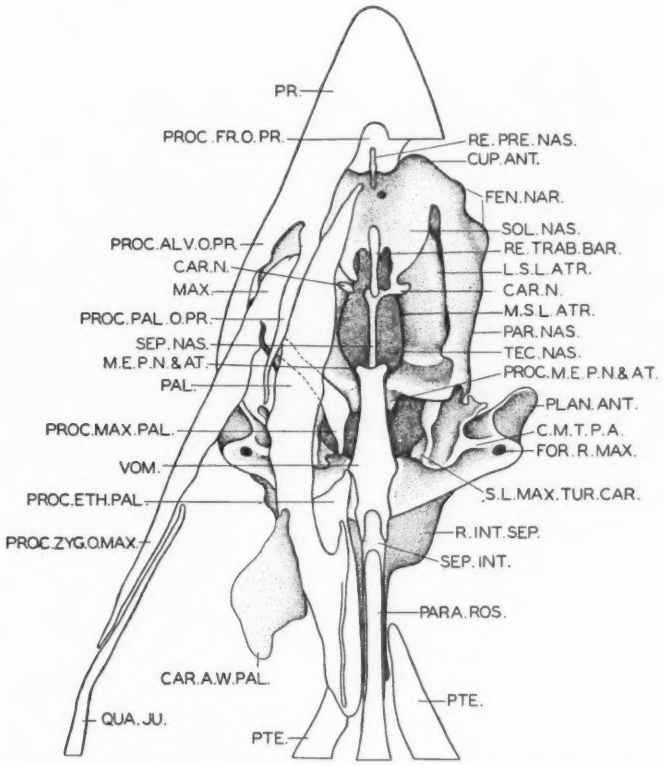

Fig. 30 ($\times = 11.5$). Stage 10. Reconstruction of the nasal capsules and the membrane bones of the anterior part of the palate (norma ventralis).

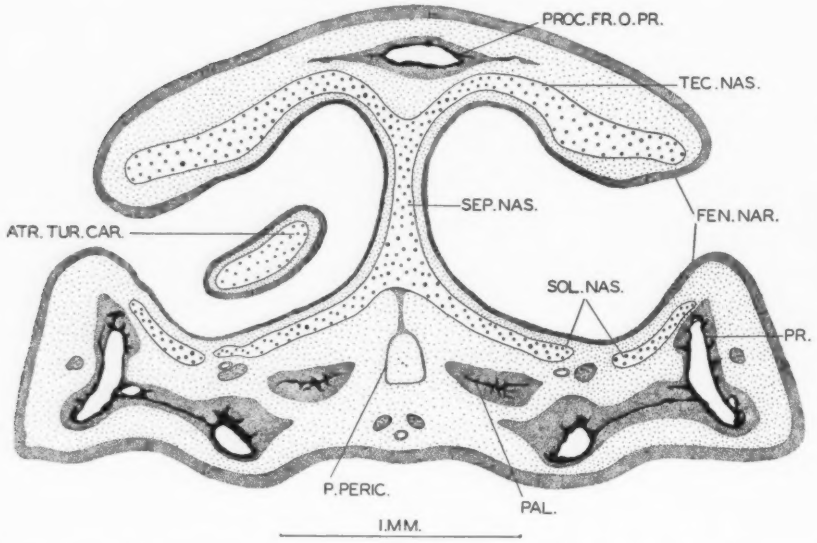

Fig. 31. Stage 10. Transverse section through the nasal capsules in the region of the fenestra narina.

facial notch which has resulted from the resorption of the trabecular bar beneath the craniofacial foramen. Anterior to the strut the original outline of the trabecula bar as well as its former continuity with the solum nasi is indicated by persisting perichondrium (Fig. 31).

Nothing now remains of the prenasal process except a part of its crest projecting antero-ventrally between the cupolae anteriores (Fig. 30). The backwardly directed processus of the solum nasi have elongated considerably, and they lie slightly below the level of the ventral edge of the septum nasi. On the one side the posterior end of the process has fused to the nodule of cartilage described in stage VIII, and this in turn has become fused to the nasal septum above the remnant of the trabecular bar (Fig. 30) by chondrification in the syndesmotic connexion existing between the nodule and the procartilaginous protuberance of the septum nasi. On the other side the nodule is still free (Fig. 32) as in stage VIII, and the process of the solum nasi terminates a short distance anterior to it.

The planum antorbitale has increased in size so that the greater part projects lateral and latero-dorsal to the side wall and roof of the nasal capsule. Its anterior surface has acquired an additional connexion with the dorsal surface of the maxilloturbinal cartilage just below the connexion between the posterior edge of the tectum nasi and the planum antorbitale. Its lateral edge is deeply notched. The posterior tip of the posteriorly directed process of its medio-

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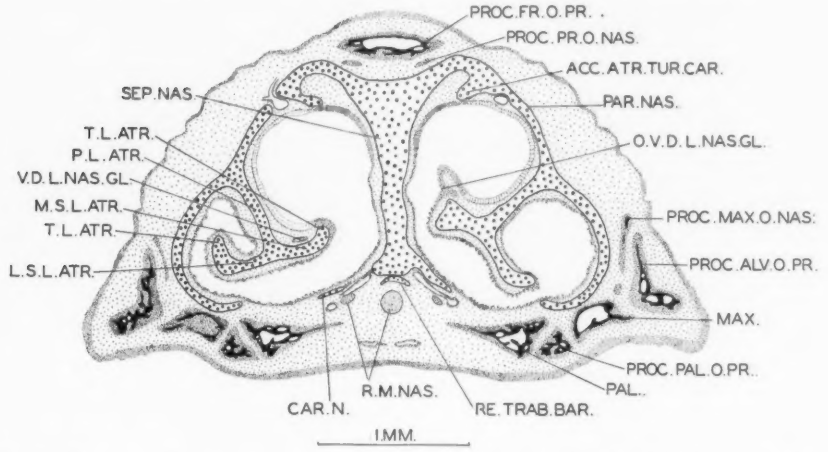

Fig. 32. Stage 10. Transverse section through the nasal capsules showing the cartilaginous nodule fused to the septum nasi.

ventral corner is completely fused to the orbital septum. No traces of vomerine cartilages are present in this stage.

Further changes in the nasal capsules affect only a few structures. The trabecular bar disappears completely leaving only a strand of connective tissue to indicate its former position. In one specimen, about to leave the nest, a small nodule of cartilage (·120 mm. in length) is present in the midline beneath the anterior end of the nasal capsules. Its presence in this specimen is probably due to a retardation of the process of resorption. In all older nestlings the small part of the crest of the prenasal process described in stage X persists.

There is considerable variation in the sequence in which the nodules of cartilage described in stage VIII fuse with the septum and with the posterior processes of the solum nasi. In a nestling of 69 mm. the processes of the solum nasi are fused with nodules, but the latter are only syndesmotically connected to the nasal septum. In a nestling of 80 mm. these nodules are synchondrotically connected to the septum nasi while the processes of the solum nasi terminates a short distance in front of them. In the oldest nestling specimen examined the processes of the solum nasi are fused with these nodules which in turn are synchondrotically connected to the septum nasi.

The vomerine cartilage first observed in stage IX reappears in a nestling of 69 mm. where it occupies the same position as in stage IX. It is still very small, only ·090 mm. in length, and it is present only on the one side. In a nestling of 80 mm. no trace of a vomerine cartilage could be found. In a specimen about to leave the nest vomerine cartilages are present on both sides as thin rods of well differentiated cartilage, each about ·465 mm. in length. Anteriorly each vomerine cartilage is continuous with the posterior process of the medial exten-

sion of the common posterior end of the atrioturbinal cartilage and side wall of the nasal capsule. Posteriorly it terminates at more or less the same place in which the separate nodule was observed in earlier stages. The vomerine cartilage chondrifies in the layer of mesenchymatous tissue along the uprising lateral edge of the vomer, and more posteriorly, medio-dorsally to the anterior tip of the dorsal lamina of the palatine. As no stages of fledglings were available it was not possible to establish the ultimate fate of the vomerine cartilages. In the adult the lateral edges of the vomer rise up high on each side to form a deep trough between them. It is possible that the cartilages have ossified in continuity with the uprising lateral edges of the vomer, as these now like in the positions formerly occupied by the vomerine cartilages.

PART II

I RÉSUMÉ AND DISCUSSION

A *The Basal Plate and Otic Capsules*

In the youngest stage the basal plate is a complete structures pierced only in the occipital region by the hypoglossal foramina. A fenestra basicranialis posterior develops in its anterior region by resorption of the cartilage as in *Tinnunculus* (SUSCHKIN, 1899). This fenestra is formed in a similar way in some *Urodela* (DE BEER, 1937). Further resorption of cartilage above the chorda results in the exposure of this structure except for the part lying in the occipital condyle. When ossification of the basal plate sets in, however, the chorda behind the fenestra basicranialis posterior is included in the ossification.

Three hypoglossal foramina on each side are the normal number in all older embryos and nestlings. In the younger embryos, however, the anterior hypoglossal root may have up to three additional foramina on the cerebral surface of the basal plate. Initially the anterior hypoglossal root has a number of twigs of which some unite inside the brain case while others enter the basal plate separately and join the rest either inside or immediately below the basal plate.

The otic capsule in the youngest stage is fused to the basal plate, but canalicular and cochlear portions can be distinguished. Of special interest is the position of the facial foramen. In the youngest stage the facial nerve is continued anteriorly through the anterior part of the cochleo-canalicular fissure. A commissure bridging the fissure is formed in front of the nerve and encloses it in a canal in the capsular wall. It does not traverse the cavity of the capsule as it is separated from it by mesenchymatous tissue that will subsequently chondrify.

The front wall of the canal for the facial nerve in *Pyromelana* is thus not homologous with the prefacial commissure of *Lacerta*, as it is not a commissure

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between the otic capsule and the basal plate, but between the two portions of the auditory capsule. The cavity of the canal is therefore not a part of the prootic fissure but part of the cochleo-canalicular fissure. SONIES (1907) pointed out that the canal for the facial nerve is situated entirely in the wall of the otic capsule. CROMPTON (1953) found that in *Spheniscus* the foramen of the facial nerve is formed between a process of the cochlear part (his processus lateralis partis cochlearis) and the canalicular part. In the older stages of *Pyromelana* the front wall of the canal increases in thickness and its medial edge then spreads over the lateral edge of the basal plate. The same condition obtains in the ostrich (FRANK, 1954) where the cartilage bordering the facial nerve foramen anteriorly is fused medially to both the cochlear part of the otic capsule and the lateral edge of the basal plate.

A process (prootic process of DE BEER and BARRINGTON, 1934) forms the posterior part of the orbitocapsular commissure in *Pyromelana*. This process grows out from the pars canalicularis in the region of the ampulla of the lateral semicircular canal and in the young nestling fuses with a posteriorly directed orbitocapsular process of the posterior part of the orbital cartilage to form the orbitocapsular commissure. SONIES (1907) recorded the presence of a prootic process in the duck and fowl. Judging from DE BEER and BARRINGTON'S (1934) illustrations, the prootic process is a conspicuous structure in the duck. In the penguin (CROMPTON, 1953) the posterior part of the orbital cartilage and the otic capsule approach each other closely, and a very short orbitocapsular commissure chondrifies in a band of procartilaginous tissue streching between them. In the ostrich the orbitocapsular commissure is equally short and is formed by a laterally directed rigde of the otic capsule (prootic process) that fuses with the posterior part of the orbital cartilage.

B *The Metotic Cartilage*

The metotic cartilage (SONIES, 1907) is a characteristic feature of the avian chondrocranium. A similar structure underlying the otic capsule is present only in crocodiles. DE BEER and BARRINGTON (1934) have pointed out that the subcapsular process of crocodiles and the metotic cartilage in birds are undoubtedly homologous. On the grounds that the subcapsular process has all the appearance of being a cranial rib and that the metotic cartilage in *Anas* appears to chondrify in relation to the cranial ribs, DE BEER and BARRINGTON (1934) considered the metotic cartilage as a probable modification of a number of cranial ribs. The ontogeny of the metotic cartilage varies from form to form, but in none of the recent investigations was there any evidence in favour of DE BEER and BARRINGTON'S contention.

T. J. PARKER (1891) was the first to mention this structure; he describes the otic capsule in *Apteryx* as as being produced laterally into a well-marked

"paroccipital process". It seems more likely that PARKER was merely describing the topography of the structure and that he did not imply its development as an outgrowth of the auditory capsule as CROMPTON (1953) maintained. According to SUSCHKIN (1899) the metotic cartilage (his "Occipitalflügel") in *Tinnunculus,* develops as a protuberance of the pars lateralis of the occipital region ("laterale Occipitalplatte"). SONIES (1907) found the metotic cartilage developing as a separate cartilaginous structure beneath the canalicular part of the otic capsule in the duck, fowl and starling. In the duck and starling the metotic cartilage first fuses with the lateral edge of the basal plate and then with the otic capsule. In some embryos of the fowls he found that fusion between the otic capsule and the metotic cartilage preceded fusion between the latter and the basal plate. DE BEER and BARRINGTON (1934), too, found a separate anlage for the metotic cartilage in the duck. BROCK (1937) described the mesenchymatous anlage of this structure in the ostrich as continuous with the tissue of the otic capsule. According to FRANK (1954) the metotic cartilage in the ostrich develops as a separate centre of chondrification. In the penguin (CROMPTON, 1953) it develops as an outgrowth of the canalicular part of the otic capsule. In these forms, then, the metotic cartilage develops either as an outgrowth of the occipital region *(Tinnunculus),* as a separate anlage *(Anas, Gallus, Sturnus* and *Struthio)* or as an outgrowth of the otic capsule *(Spheniscus).*

In *Pyromelana* it develops from a medial as well as a lateral anlage. The medial anlage is apparently an outgrowth of the occipital region. In stage I it is present as a procartilaginous plate projecting from the antero-lateral corner of the pars lateralis of the occipital region. The procartilaginous lateral anlage occupies the same position as the anlage of the metotic cartilage in *Anas, Gallus, Sturnus, Struthio* and *Spheniscus.* Its ontogeny is different, however, in that it does not appear as a separate element nor as an outgrowth of the otic capsule. In the youngest stages the lateral anlage is included in the thickened lateral part of the floor of the canalicular part of the otic capsule. It is only after it has chondrified to some extent and after resorption of intercellular substance between it and the floor of the otic capsule has taken place that it can be discerned as a separate structure. Fusion between the anlagen takes place when both are still procartilaginous and the lateral anlage is still closely attached to the floor of the auditory capsule. Upon chondrification that part of the metotic cartilage chondrifying in the lateral anlage becomes separated from the capsular floor, except anteriorly, where for a short distance its anterior edge is fused to the auditory capsule in the region of the ampulla of the lateral semicircular canal. This partial discontinuity is only temporary: in the nestling its lateral and posterior edges will fuse completely to the prominence of the lateral semicircular canal and more medially to the prominence of the posterior semicircular canal, thus closing the cavum metoticum laterally and postero-laterally.

The procartilaginous connexion which has always existed between the poste-

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rior edge of that part of the metotic cartilage chondrifying in the medial anlage and the prominence of the superior semicircular canal becomes chondrified in the nestling.

The medial edge of the medial anlage fuses early during the development with the lateral edge of the basal plate just anterior to the vagus-accessory nerve, thus converting part of the fissura metotica into a foramen for this nerve. The glossopharyngeal nerve leaves the cranial cavity through the anterior part of the fissura. The latter has been delimited posteriorly by the fusion of the posterior edge of the medial anlage of the metotic cartilage to the otic capsule.

The process of the ventral surface of the metotic cartilage lying ventro-lateral to the cavity of the middle ear and posteriorly and laterally to the hinder part of the meatus acusticus externus has also been described in *Tinnunculus* (Suschkin, 1899). Sonies (1907) described similar processes in the duck and fowl but as his description is somewhat incomplete, it is not possible to ascertain the extent of these processes in relation to neighbouring structures.

C *The Orbital Cartilage*

Although the early development of the orbital cartilage has been thoroughly described by most authors, very little attention has been given to the fate of the structure in the nestlings. Some degree of resorption in the older stages was noted by everyone, but in most cases investigation was not carried far enough to establish its ultimate fate.

The usual manner of development is that the anterior part develops in continuity with the dorsal edge of the interorbital septum while the postorbital part develops in continuity with the pila antotica. In *Larus* (de Beer and Barrington, 1934), however, the orbital cartilage has an independent centre of chondrification, while in *Gallus* (Sonies, 1907) the posterior part appears as a separate centre. Separate anlagen for parts of the orbital cartilages have been described by Suschkin (1899) in *Tinunculus* as orbitosphenoids; they fuse with the dorsal edge of the interorbital septum. De Beer and Barrington (1934) described a pair of similar structures in the duck and regarded them as probably representing the taeniae mediales of reptiles.

In those forms in which a complete orbital cartilage is formed, both the anterior and posterior parts are prolonged above the eye where they fuse; this middle portion is then referred to as the supraorbital part. In the forms thus far investigated a complete orbital cartilage stretching from the interorbital septum to the pila antotica occurs in *Anas* (de Beer and Barrington, 1934), *Struthio* (Brock, 1937 and Frank, 1954), *Ardea* (Slabý, 1952), *Spheniscus* (Crompton, 1953) and *Melopsittacus* (Lang, 1954). In *Pyromelana* a complete orbital cartilage does not develop.

The anterior part of the avian orbital cartilage fuses to the lateral surface of the dorsal part of the interorbital septum enclosing the olfactory nerve in a long canal. The only bird known in which such an olfactory canal is not formed is *Spheniscus* (CROMPTON, 1953). DE BEER (1937) stated that in birds the large dimensions of the eyes bring about this condition (e.g. in *Passer*). The anterior parts of the orbital cartilages are plastered, as it were, against the interorbital septum. The experiments of BELLAIRS (1955), however, clearly showed that the size of the eyeball has no effect on the development of the neighbouring parts of the chondrocranium. Ablation of one eye in early chick embryos had no effect on the development of the anterior part of the orbital cartilage. He found the same condition in a naturally occurring anomalous chick embryo in which one eye was rudimentary.

The inclusion of the olfactory nerve in a canal is only temporary, for later during the ontogeny the anterior part of the orbital cartilage is resorbed so that the olfactory nerve traverses the orbit. In *Pyromelana* the anterior part of the orbital cartilage disappears completely in the nestling. According to DE BEER and BARRINGTON (1934) part of the anterior orbital cartilage in the duck remains attached to the dorsal edge of the interorbital septum as a planum supraseptale. The supraorbital part is resorbed in all forms in which the development has been followed. Also resorption along the anterior edge of the posterior part seems to be the rule.

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The pila antotica spuria which is the main structure connecting the posterior part of the orbital cartilage to the basal plate is a stout structure in the nestling of *Pyromelana*. It appears early in the ontogeny between the ventral edge of the posterior part of the orbital cartilage and the lateral edge of the basal plate just lateral to the profundus nerve. It gradually increases in thickness and takes over the support of the posterior part of the orbital cartilage as the pila antotica is being gradually resorbed along its anterior and medial edges. In stage VIII the pila antotica is only a thin strip of cartilage lying medially to the profundus nerve. In *Gallus*, according to DE BEER and BARRINGTON (1934), the pila antotica disappears completely and only a pila antotica spuria remains.

D *The Processus Basipterygoideus and the Infracarotid Commissure*

The term quadratopolar commissure is being introduced in this paper to designate the transitory procartilaginous connexion existing between the pars quadrata palatoquadrati and the polar cartilage in the younger stages. A similar commissure has been described for the pigeon (FILATOFF, 1906) and the duck (DE BEER and BARRINGTON, 1934). LANG (1952), in a paper on the development of *Melopsittacus*, *Gallus* and *Columba*, stated that a connexion between the pars quadrata and the neurocranium is present in the younger stages, but she did not comment upon its morphology. In *Pyromelana* the commissure develops as an

outgrowth of the pars quadrata, and its tip fuses with the antero-ventral corner of the polar cartilage. FILATOFF (1906) regarded this commissure (his columella) as a continuation of the pars quadrata.

In *Pyromelana* resorption of intercellular substance takes place in the commissure some distance lateral to the point where the palatine nerve passes forwards below it. The portion of the quadratopolar commissure dorsal to the point of resorption chondrifies as the pr. basipterygoideus. FILATOFF (1906) described a similar discontinuity in the quadratopolar commissure of the pigeon. The dorsal part of the commissure helps to bound the lateral carotid foramen. It has been pointed out above that the proximal portion of the pr. basipterygoideus in *Pyromelana* forms the anterior part of the ventral border of the lateral carotid foramen. In *Anas* the quadratopolar commissure disappears completely, and a pr. basipterygoideus is absent.

Since the pr. basipterygoideus of *Pyromelana* chondrifies in the dorsal portion of the quadratopolar commissure, it is of visceral origin, and its connexion to the neurocranium is secondary. CROMPTON (1953) and FRANK (1954) maintained that in *Spheniscus* and *Struthio* the pr. basipterygoideus (their pr. basitrabecularis) arises autochthonously and then chondrifies in continuity with the neurocranium. CROMPTON'S (1953) statement that in Fig. 92 of T. J. PARKER'S (1891) description of the development of the *Apteryx* skull there is an independent anlage for the pr. basipterygoideus (Crompton referred to it as a pr. basitrabecularis while Parker labelled it basipterygoid process) is patently incorrect, because the process is clearly shown as being continuous with the trabecula.

The pr. basipterygoideus is a conspicuous feature of the older embryos and young nestlings of *Pyromelana,* and it even ossifies to some extent. In the older nestlings resorption of the structure sets in, and in the adult no trace of it is left. It never articulates with the pterygoid.

The term infracarotid commissure is being introduced in this paper to designate the commissure between the prechordal region and the basal plate; it forms the ventral border of the lateral carotid foramen. There is considerable difference in the mode of formation of this structure in the various forms in which its development has been studied. In *Pyromelana* the anterior portion of the commissure is formed by the backwardly directed proximal part of the pr. basipterygoideus, and the posterior by an anteriorly directed protuberance of the basal plate. The medial portion is formed by the chondrification of a strand of mesenchymatous tissue stretching between the tip of this protuberance and the medial edge of the proximal part of the pr. basipterygoideus. An infrapolar process which forms this commissure in the fowl and starling (SONIES, 1907), the duck (SONIES, 1907 and DE BEER and BARRINGTON, 1934) and the ostrich (BROCK, 1937 and FRANK, 1954) is not present in *Pyromelana*. In the ostrich FRANK (1954) described the fusion of the tip of the infrapolar with the basal

plate by means of a protuberance of the latter. In the penguin (CROMPTON, 1953) the infracarotid commissure is formed in an entirely different manner. The ventral edge of the acrochordal migrates forwards and fuses with the ventral portion of the basipterygoid process (basitrabecular process of Crompton).

When the cephalic flexure is straightened out this connexion is stretched to form the ventral border of the lateral carotid foramen.

The terms pr. basipterygoideus and pr. basitrabecularis have been used in different senses by the authors of the 20th century, and this has resulted in much confusion when it came to a comparison of the results of these workers. The name pr. basitrabecularis (Basalfortsatz der Trabekel) was given by SUSCHKIN (1899) to a process of the posterior end of the trabecula projecting posteriorly beneath the basal plate in *Tinnunculus*. The distal tip of the process fuses with the basal plate to complete the ventral border of the lateral carotid foramen. The pr. basipterygoideus develops as a protuberance along the lateral edge of the pr. basitrabecularis. The meaning of Suschkin's term is quite clear: it is supposed to be a process of the trabecula directed towards the basal plate. SONIES (1907) proposed the name infrapolar process for Suschkin's basitrabecular process, as he could establish clearly that this process developed from the polar cartilage. (Suschkin did not see the polar cartilages in *Tinnuncules.*) Sonies did not describe a basipterygoid process in the forms studied by him.

Suschkin's terminology, however, was retained by LUTZ (1942) who described both basipterygoid and basitrabecular processes in *Dromiceius*. Other authors referred to this structure as the infrapolar process. DE BEER and BARRINGTON (1934), BROCK (1937), CROMPTON (1953) and FRANK (1954) have described the pr. basipterygoideus in the forms studied by them as a pr. basitrabecularis. According to DE BEER and BARRINGTON (1934) a basitrabecular process is a laterally directed process of the polar cartilage which meets, fuses with, or is articulated with the basal process of the pars quadrata. It is obvious that the process described by them is not the pr. basitrabecularis as defined by SUSCHKIN (1899).

When therefore later authors compared the development of their basitrabecular process (the pr. basipterygoideus) with the development of the pr. basitrabecularis of SUSCHKIN (1899) or LUTZ (1942) they compared completely different morphological structures.

E *The Nasal Capsules*

With the possible exception of the planum antorbitale the whole nasal capsule develops from the trabecula communis. The septum nasi develops as an upgrowth of the dorsal edge of the trabecula communis, while the roof and side wall develop as an outgrowth along the dorsal edge of the turbinals and then

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grow out from these structures. W. K. PARKER (1876) described the roof, side walls and turbinals in *Gallus* as developing in the form of outgrowths of the dorsal edge of the septum nasi. In line with this are also the observations of BORN (1879) on *Gallus* and of SUSCHKIN (1899) on *Tinnunculus*. These authors stated that the roof and side walls make their appearance as condensations of connective tissue along the dorsal edge of the nasal septum. When chondrification sets in, it proceeds from the nasal septum outwards. GAUPP (1906) held the same view concerning the origin of these structures. SONIES (1907) claimed an independent anlage for the side wall and turbinals in the duck, fowl and sparrow. This anlage subsequently fuses with the roof which develops as an expansion of the dorsal edge of the septum. According to DE BEER and BARRINGTON (1934) the roof and side wall of the capsule in the duck are formed by the extending dorsal edge of the nasal septum.

As in *Gallus* (BORN, 1879) and *Tinnuncules* (SUSCHKIN, 1899), the tectum nasi develops from behind forwards. SONIES (1907) claimed that in *Anas* and *Gallus* the direction of development is exactly the opposite. No anlage of a paranasal cartilage, as described by DE BEER and BARRINGTON (1934) in *Anas* and FRANK (1954) in *Struthio* nor any structure comparable to a concha nasalis, was found in *Pyromelana*. According to SCHULTZE (1862) a concha nasalis is absent in *Columba, Psittacus* and numerous "Singvögeln" (= *Passerinae*).

The planum antorbitale develops as a separate centre of chondrification within a concentration of mesenchymatous tissue continuous anteriorly with the mesenchymatous plate in which the roof of the capsule chondrifies. When it appears for the first time as a procartilaginous structure, no intercellular substance is present in the band of mesenchymatous tissue connecting it to the posterior edge of the procartilaginous tectum nasi. A synchondrotic continuity between the two structures is only established when both have become hyaline.

It was not possible to establish whether the mesenchymatous anlage of the planum antorbitale developed as an outgrowth of the tectum nasi or as a local condensation of mesenchyme secondarily continuous with the mesenchymatous anlage of the tectum nasi.

PARKER and BETTANY (1877) claimed that in *Gallus* the planum antorbitale ("antorbital plate") develops from the posterior part of the roof of the nasal capsule ("aliethmoidal roof"). DE BEER and BARRINGTON (1934) described a separate anlage for this structure in *Anas*, and SLABÝ (1952) figured it as a separate entity in *Ardea*. In *Spheniscus* (CROMPTON, 1953) the planum antorbitale develops out of the lower posterior region of the side wall of the nasal capsule. Unfortunately DE BEER and BARRINGTON do not mention the relation of the mesenchymatous anlage of the planum antorbitale to the rest of the capsule, so that as far as the origin of the anlage is concerned in forms where the planum antorbitale chondrifies as a separate anlage, we are still completely in the dark.

Everyone seems to agree that the turbinal cartilages develop from the roof and side wall, the atrioturbinal cartilage as a ridge on the ventral surface of the roof in the anterior region of the capsule and the maxillo-turbinal cartilage by an inrolling of the ventral edge of the posterior part of the side wall. In the genera which I have examined there is much variation in the degree of complexity of the atrioturbinal cartilage. In *Nyctisyrigmus, Spheniscus* and *Anas,* where the nasal capsules are very long, the atrioturbinal cartilages are much simpler than in *Pyromelana,* where the capsules are much shorter. In *Nyctisyrigmus* they are simple ridges, and in *Spheniscus* and *Anas* they bear short lamellae. The atrioturbinal cartilage in *Pyromelana* bears two large secondary lamellae, each of which bears short tertiary lamellae. According to GIEBEL (1876) the atrio-turbinal cartilages are very complicated in the *Passerinae.* W. K. PARKER (1875, 1876, 1878) refers to several forms possessing large atrioturbinal cartilages. Of special interest is his observation of the presence of large atrioturbinal cartilages in the South African Ploceinid, *Estrelda astrild.* Atrioturbinal cartilages are absent in *Podargus* and *Gypogeranus* (GEGENBAUR, 1873).

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The atrioturbinal cartilage is for the greater part covered by thick keratinized epithelium, so that it is difficult to ascribe any function to this structure except to its posterior part which is covered by respiratory epithelium. STRESEMANN (1927—34) considered it probable that its function is to prevent the entrance of foreign substance into the nasal cavity. In connexion with the atrioturbinal cartilage it may be mentioned that in *Nyctisyrigmus* the tissue underlying the keratinized epithelium is intensly vascularized. This vascularization also occurs in the tissue of the floor of the nasal cavity and on the maxilloturbinal cartilage. The blood supply is quite out of proportion to the amount of tissue in this region. It is perhaps not yet possible to explain the function of this nasal erectile tissue.

In *Pyromelana* the common posterior edges of the side walls as well as the atrioturbinal cartilages extend medially to clasp the anterior end of the trough-like azygous ossification of the vomer between them. In the adult these medial extensions ossify in continuity with the anterior part of the vomer. In his two papers on "aegithognathous birds" dated 1875 and 1879, W. K. Parker recorded similar outgrowths of the nasal walls and of the atrioturbinal cartilages; he also observed their ossification in continuity with the vomer in a large number of forms.

W. K. PARKER and BETTANY (1877) described in the young fowl the posterior en of the atrioturbinal cartilage as growing inward and becoming connected to the nasal septum by the intervention of a process of the latter pierced by the n. ramus lateralis nasi. This process was first observed as a ridge situated near the bottom of the septum, and covering the n. ramus lateralis nasi dorsally. Judging from Tonkoff's model of the skull of the fowl the connexion of the

posterior end of the atrioturbinal cartilage with the septum takes place dorsal to the trabecular bar. It seems likely that these processes in the fowl are homologous with a pair of similar structures described in the duck by DE BEER and BARRINGTON (1934) and SWART (1946).

They project ventro-laterally from the nasal septum, some distance above the trabecular bar and roof the median branches of the nasal nerve, but they are fairly distant from the posterior ends of the atrioturbinal cartilages. It is very unlikely that these processes in the duck are homologous with paraseptal I of *Crypturellus* (DE VILLIERS, 1946) as SWART (1946) suggested. In *Crypturellus* paraseptal I which de Villiers regards as an unossified tract of the ventro-lateral rim, is cemented to the base of the nasal septum which is formed by the ossified trabecular bar, as this structure is not resorbed in "palaeognathic" birds. In *Anas,* as already pointed out, they project from the septum dorsal to the trabecular bar. Swart's main argument in favour of their homology in the two forms: that in both cases these structures belong to the ventral edge of the nasal septum, is based on the erroneous assumption that the part of the trabecular bar beneath these processes in the duck is a posterior prolongation of the prenasal process.

The maxilloturbinal cartilage in *Pyromelana* is a simple structure and bears secondary lamellae near its posterior end. Posteriorly it has two synchondrotic connexions with the anterior surface of the planum antorbitale. It does not ossify in the adult, and its whole surface bears respiratory epithelium.

A floor for the anterior part of the vestibular region of the nasal sac develops as a backgrowth of the ventral edge of the cupola anterior. DE BEER and BARRINGTON (1934) and SWART (1946) described similar processes in the embryonic nasal capsule of the duck. In the oldest stage described by Swart the floor is incomplete, as the medial edge is not fused to the nasal septum. A complete floor for the anterior part of the nasal sac, as in *Pyromelana,* is uncommon in birds and is described only in falcons (W. K. PARKER and BETTANY, 1877, and STRESEMANN, 1927—1934). SUSCHKIN (1899) described a floor in this region for *Tinnunculus.* From his description it appears that in *Tinnunculus* the floor is formed by an ingrowth and subsequent fusion of the ventral edge of the side wall with the nasal septum. Judging from Stresemann's (op. cit.) account of the nasal capsules, this mode of origin applies to the *Falconidae* as a whole. Unfortunately Suschkin did not describe at which point the fusion between the floor and the nasal septum takes place. In *Pyromelana* the medial edge of the posteriorly directed process of the cupola anterior fuses with the septum some distance above the trabecular bar. LANG (1954) described a floor for the anterior region of the capsule in *Melopsittacus undulatus;* it is supposed to be formed by the paranasal cartilage, but this seems very improbable.

The fenestra narina becomes surrounded by cartilage as the result of fusion of the antero-ventral corner of the parietotectal cartilage with the lateral edge

of the solum nasi. A fenestra narina completely surrounded by cartilage has been described only for *Falco tinnunculus* (PARKER, 1876), *Tinnunculus alaudarius* (SUSCHKIN, 1899) and *Passer domesticus* (DE BEER and BARRINGTON, 1934).

The fate of the posterior processes of the solum nasi has has already been discussed, and it was pointed out that they became connected to the nasal septum by the intervention of separate cartilaginous nodules. Exactly the same condition obtains in the duck. DE BEER and BARRINGTON (1934) and SWART (1946) found a pair of antero-lateral processes in their 17 and 16 day stages respectively. Judging from their illustrations these are processes of the trabecular bar. SWART (1946) described them as processes of the prenasal process, as he, following DE BEER and BARRINGTON (1934), erroneously held this part of the trabecular bar to be a posterior prolongation of the prenasal process. I have observed the anlagen of these processes in an 11 day embryo of *Anas* as struts of condensed mesenchymatous tissue containing faint traces of intercellular deposit. Their postero-dorsal ends are closely applied to the lateral surfaces of the trabecular bar, but a well-developed perichondrium is discernible between the dorsal end of each process and the trabecular bar.

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As the latter already possesses a well-developed perichondrium in a 10 day embryo, it is obvious that these processes do not develop as outgrowths of the trabecular bar but from separate anlagen, exactly as in *Pyromelana*. In a 16 day embryo, such anlagen are procartilaginous, but the intervening perichondrium between their dorsal tips and the trabecular bar is still discernible, thus proving that they arise autochthonously. In a 20 day embryo they are completely chondrified, and no sign of an earlier discontinuity of matrix between them and the trabecular bar can be detected. Their anterior tips are fused to the posteriorly directed processes of the cupolae anteriores. In both *Pyromelana* and *Anas* the posterior termination of the floor is indicated by a separate cartilaginous nodule which later becomes connected to the nasal septum and with the rest of the floor in front of it. In *Pyromelana* the fusion with the nasal septum takes place above the trabecular bar, while in *Anas* it takes place with the trabecular bar itself. In the adult the solum nasi ossifies in continuity with the palatal parts of the premaxillaries and the anterior ends of the palatines. The presence of a long prenasal process is a characteristic feature of all avian chondrocrania. It is transitory and disappears more or less completely in the older stages. W. K. PARKER (1866) regarded the prenasal process as being formed by the confluence of the trabeculae.

In a later publication, dated 1869, he changed his mind, and both Parkers then contended that the prenasal process (prenasal cartilage, prenasal rostrum, intermaxillary axis or snout cartilage) is formed by the so called "intertrabecula". SUSCHKIN (1899) held the same view. SONIES (1907) expressed his doubts as to whether the procartilaginous tissue between the trabeculae which

Suschkin had described as an intertrabecula could be regarded as such. He was unable to observe an "intertrabecula" in either the duck, fowl or sparrow. In these forms the processus prenasalis is formed as an anterior prolongation of the cartilage connecting the trabeculae. The observations of later investigators as well as the results of the present investigation show that the idea of a separate element between the trabeculae, giving rise to the prenasal process, is no longer tenable.

The theory of a prenasal process growing out of the trabecula communis, seems to be prevalent among later investigators, but it gives no explanation of the phylogeny of the structure and leads to complicated and erroneous explanations of conditions in the anterior region of the nasal capsules, especially in older stages. Considering the circumstances that this process is transitory and subsequently more or less completely disappears, it seems improbable that it is a definite active outgrowth of the trabeculae.

W. K. Parker's explanation, that it serves as a substratum around which the membrane of this region are formed seems very unlikely. The presence of a process projecting in front of the nasal capsules in birds, crocodiles, lizards (*Varanus, Chamaesaura* and the *Iguanidae* (MALAN, 1946) and *Echidna* suggests another explanation of the presence of this structure. The prenasal process should not be looked upon as a special process developing in front of the nasal capsules, but as the original anterior part of the trabecula communis; and its position in front of the nasal capsules must be due to the front walls of the latter being displaced posteriorly. The development of the prenasal process, described as an outgrowth of the anterior end of the trabecula communis, is nothing more than the normal prolongation of the trabecula communis in order to keep up with the enlargement of the head as a whole.

The circumstances that during the ontogeny of birds a transitory part of the trabecula communis projects in front of the nasal capsules can best be explained in the light of the phylogeny of the class. As LOWE (1944) has remarked, birds in contrast to their reptilian predecessors have their premaxillaries very much enlarged. In reptiles the premaxillaries form a mere "capping" for the snout, and the external nares are usually situated at the very tip of the snout. In all birds, to my knowledge (except in *Apteryx)* the external nares have been carried posteriorly by the enlarging premaxillaries. It follows quite logically that the enlargement of the premaxillaries and the formation of a horny bill has resulted in the posterior displacement of the anterior part of the nasal capsules, and that in birds the premaxillaries now lie in a position once occupied by the anterior part of the nasal capsules in their reptilian predecessors. This posterior displacement of the external nares is already present in *Archaeornis* where the premaxillaries are already much more elongated anterior to the external nares than in *Euparkeria* or *Aëtosaurus.*

In the ontogeny of birds the anterior wall of the nasal capsules is no longer

formed at the anterior tip of the trabecula communis, but some distance posterior to it, so that what appears to be a process growing out of the trabecula communis in front of the nasal capsules is nothing but the normal anterior prolongation of the trabecula communis keeping pace with the enlargement of the head. This anterior part of the trabecula communis or prenasal process thus lies in a position later to be occupied by the premaxillaries, and as the latter ossify, the prenasal process is resorbed.

Evidence in favour of this explanation of the presence of a prenasal process in birds caused by the effects of the enlargement of the premaxillaries is furnished by conditions obtaining in *Apteryx*.

In this genus elongation of the nasal and palatine processes of the premaxillaries took place as in all other birds, but in such a manner that the external nares were not shifted posteriorly but retained a subterminal position. In comparison with other birds a very small part of the fused premaxillaries lies in front of the external nares. The ontogeny reveals no part of the trabecula communis projecting in front of the walls of the nasal capsules in this genus. The Parkers were mistaken when they regarded the anterior free portion of the nasal septum between the anterior parts of the capsular walls as a prenasal process. The term prenasal process is only applicable when part of the trabecula communis projects in front of the walls of the nasal capsules. As the anterior part of the nasal capsules has not been displaced posteriorly, no part of the trabecula communis will project in front of the nasal capsules. The absence of a prenasal process in *Apteryx* is thus not a secondary feature resulting from the elongation of the snout but represents the original reptilian condition. This raises the important point: in how far is *Apteryx* really primitive, i.e. more "reptilian" than other "Dromaeognathae"?

The presence of a prenasal process in the crocodile can perhaps be accounted for by the circumstances that the nostrils are dorsally as well as posteriorly displaced. In *Varanus* too, the nostrils are situated very far back. In all forms (birds, various reptiles and *Echidna*), where a prenasal process is present it should be regarded as the anterior tip of the trabecula communis projecting in front of the nasal capsules and as a special modification in the anterior region of the snout. It should not be regarded as a special process developing in these forms alone, or as possessing any morphological significance.

Malan (1946) did not regard the presence of a prenasal process in the *Iguanidae* and *Varanus* as proof of any affinity between the latter and the *Ascalabota* but considered it as a Rhynchocephalian character of some primitive Lacertilian families. It is very doubtful whether the presence of a prenasal process can be regarded as a primitive characteristic at all; it is more probable that its presence in these various reptilian groups is due to parallel modifications of the anterior part of the snout.

De Beer and Barrington (1934) and Swart (1946) regarded that portion

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of the trabecular bar in *Anas* extending up to the craniofacial fenestra as the base of the prenasal process. They gave no reasons for their conclusion nor did they describe any backward growth of the prenasal process during ontogeny. An examination of a development series of *Anas* gave me no reason to regard this part of the trabecular bar as part of the prenasal process as the ontogeny is absolutely identical with that of *Pyromelana*.

The craniofacial fenestra and the fenestra septalis develop as the result of resorption of the nasal septum above the trabecular bar. Complete resorption of the trabecular bar in the nestling results in the disappearance of the fenestra septalis and converts the craniofacial fenestra into a craniofacial notch. SWART (1946) described an anterior and a posterior fenestra septalis in *Anas* and stated that the fenestra septalis posterior is converted into a craniofacial notch by the resorption of the "prenasal process". (He erroneously regarded this part of the trabecular bar as the base of the prenasal process.) The only fenestra in the nasal septum of adromoeagnathic birds that can be homologized throughout is the one that is always ultimately converted into a craniofacial notch; the number of fenestrae in front of this varies. CROMPTON (1953) described two septal fenestrae anterior to the craniofacial fenestra in the *Spheniscus*. I have found only one in *Pyromelana, Nyctisyrigmus* and *Anas*. What SWART (1946) described as a fenestra septi nasi posterior is really the craniofacial fenestra.

It is very doubtful whether the vacuity in the nasal septum found by T. J. PARKER (1891) in only one stage in the development of *Apteryx*, and which he considered as probably homologous with the craniofacial notch of the fowl, can be regarded as such. From what can be made out from his description and illustrations it is a cavity completely enclosed in the nasal septum and not a fenestra. It is thus improbable that this vacuity represents a transitory rudiment of the craniofacial notch, as also SUSCHKIN (1899) maintained, or, that it lends any support to the view that the *Ratitae* can be regarded as degenerate *Carinatae*. An important feature of the ethmoidal region (and one that has been neglected by all investigators except W. K. Parker) is the resorption of thre trabecular bar in the older stages of "neognathic" birds. It has already been pointed out that in *Pyromelana* the prenasal process together with the trabecular bar in the ethmoidal region has been completely resorbed by the time the chick leaves the nest. Together with the trabecular bar the part of the septum nasi between the trabecular bar and the solum nasi is also resorbed. I have found similar resorption of the trabecular bar in *Spheniscus* and *Nyctisyrigmus*. W. K. Parker, though he mentioned resorption of the trabecular bar in various forms, did not record the extent of such resorption. It is obvious however, that in all "neognathic" birds some resorption must take place in order to convert the craniofacial fenestra into a craniofacial notch. In "palaeognathic" birds resorption of the trabecular bar, except for the prenasal process, does not occur. In the tinamu resorption of the trabecular bar beneath the extreme anterior part

of the nasal capsules has apparently taken place. In an adult tinamu *(Crypturellus* species) no trabecular bar is present beneath the extreme anterior end of the nasal capsule. As the ontogeny of the tinamus is not known it is impossible to say whether its absence in this region is due to resorption or not.

A thickened trabecular bar in the ethmoidal region is a characteristic feature of all avian chondrocrania. Among living reptiles thickening of the ventral part of the nasal septum is never as extreme as in birds. In *Lacertilia* and *Ophidia* it is either absent or hardly noticeable. Among the akinetic orders the ventral part of the septum nasi is thickened, in crocodiles almost to the same extent as in birds. In the reptilian stock from which the birds have developed there must have been a tendency for the ventral part of the septum nasi to increase in size, such increase probably having taken place in order to provide rigidity for the elongating snout region.

This would entail a decrease in the flexibility of the ethmoidal region and thus a restriction of kinesis. If kinesis was to be retained in birds, as is actually the case, certain structural alterations had to take place in the ethmoidal region in order to overcome this lack of flexibility. De Villiers (1946) first drew attention to the alternative requirements for mesokinesis in birds, namely, either "fracturing" of the septal apparatus or "lifting off" from the nasal capsules, of the membrane bones of the nasal region. "Fracturing" of the septum occurs in all "neognathic" birds while "lifting off" of the dermal bones takes place in "palaeognathic" birds with the exception of *Apteryx* which, according to de Villiers (1946), is probably akinetic. De Villiers (1946) regarded the alterations conditional to avian mesokinesis as due to the greater degree of incorporation of the nasal capsules into the bony braincase as compared with living reptiles, such as lizards. I am of the opinion that the primary reason for these alterations was the loss of flexibility of the ethmoidal region caused by the increase in thickness of the trabecular bar. If ossification of the ethmoidal region had set in very early in the phylogeny of the class, it would no doubt have been partly responsible for the loss of flexibility of the ethmoidal region and thus partly responsible for the necessity of structural alterations in this region.

If we assume that the succession of events in the ethmoidal region of a "neognathic" bird, namely an initial formation of a complete nasal septum and thickened trabecular bar and a consequent resorption of the trabecular bar and part of the septum, is an accurate recapitulation of the phylogeny, the condition in "palaeognathic" birds, where no such resorption occurs, must be regarded as representative of the original avian condition. Both "palaeognathic" and "neognathic" types have apparently developed from a common stock in which the trabecular bar was thickened and complete. In each type the impediment to kinesis brought about by this structure has been overcome in a different way. It is difficult to say whether the elimination of the restriction of kinesis along two different lines effected by the increase in size of the ventral part of the

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nasal septum proceeded simultaneously with the development of that structure, or whether elimination only set in after the trabecular bar had increased in thickness to such an extent as to hamper kinesis. The circumstance that a thickened trabecular bar is recapitulated to my knowledge very clearly in all "neognathic" types seems to be more in favour of the latter possibility. In that case the postulation of a stage in the phylogenetic history of the class, in which cranial kinesis was more restricted than in modern birds, seems inevitable. Interesting in this connexion are statements by HEILMANN (1926) and STRESEMANN (1927—1934) that the skull of *Archaeornis* was probably akinetic.

F *The Vomerine Cartilages*

The term "vomerine cartilages" was introduced by W. K. Parker in a paper read before the Linnean Society of London on December 16th, 1875. This paper was published in the following year but the date of the bound volume is 1879. In this paper Parker stated that he had discovered in the Brown Linnet *(Linota cannabina)* structures for which he proposed the name "vomerine cartilages", and that they were completely new to him. It is rather strange then to find that, in a paper read by him before the Zoological Society of London on February 18th, 1873, he described these structures by name in a number of birds. The paper was only published in December, 1875 (the date of the bound volume is 1877), that is in the same month and year as the announcement of the discovery of these structures in the paper read before the Linnean Society of London. Unless the papers were dated incorrectly the only possible explanation for the discrepancy is that Parker, after having discovered these structures in *Linota cannabina,* looked for them in the forms dealt with in the paper read in 1873, and added descriptions of the vomerine cartilages to his original paper before publication in 1875.

After T. J. PARKER (1888) had discovered similar structures in *Apteryx* both Parkers regarded them as remnants of the cartilaginous capsules of Jacobson's organs. When W. K. PARKER (1888) put forward this view he also stated that he had observed the structures in *Rhea* as early as 1866 but that he had not understood their nature at the time. DE BEER and BARRINGTON (1934) described a pair of "prevomer cartilages" in *Anas.* In the general section on the nasal capsules in his textbook, DE BEER (1937) stated that among birds remnants of paraseptal cartilages are apparently preversed in *Apteryx,* (Jacobson's cartilages of T. J. Parker), but he did not mention the "prevomer cartilage" of *Anas* in this connexion. SWART (1946) regarded the structures in *Anas* described by DE BEER and BARRINGTON (1934) as paraseptal cartilages (his paraseptals II). DE VILLIERS (1946) described three paraseptals on each side in *Crypturellus;* of these he considered paraseptals II and III to represent parts of the solum nasi. His conclusions were based upon the study of adult material.

The vomerine cartilages in *Pyromelana* make their appearance in the nestling as small nodules of cartilage dorso-medial to the dorsal edge of the anterior tip of the dorsal lamina of the palatine bone.

In the oldest nestling investigated each vomerine cartilage is a thin, rodshaped structure. Its posterior portion has the relations to the anterior tip of the dorsal lamina of the palatine described above, while its anterior part is situated along the uprising lateral edge of the troughshaped azygous vomer. Its anterior tip is fused to the posteriorly directed process of the medial extension of the common posterior edge of the atrioturbinal cartilage and the side wall of the nasal capsule. In the adult they have apparently ossified in continuity with the vomer.

In *Anas* vomerine cartilages appear early in ontogeny. Their mesenchymatous anlagen can be observed for the first time in an embryo of 7½ days, as two struts of mesenchymatous tissue attached to the ventro-lateral edges of the trabecular bar. DE BEER and BARRINGTON (1934) do not figure them before the 14 day embryo, but in the series of *Anas* in this Institute they are already chondrified and separated from the trabecular bar in the 10½ day embryo. They are situated some distance in front of the azygous vomerine ossification, the first traces of which can be seen in this stage. In subsequent stages the vomer is continued anteriorly below the vomerine cartilages.

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When the development of the vomerine cartilages in *Anas* and *Pyromelana* is compared, a number of differences emerge. In *Pyromelana* the appearance of the vomer precedes that of the vomerine cartilages. These develop in the nestling. But in *Anas* the cartilages develop very early; mesenchymatous anlagen are present in the 7½ day embryo when there is still no trace of an anlage of the vomer. A mesenchymatous anlage for the latter cannot be detected before the 9½ day stage when intercellular deposit in the anlagen of the vomerine cartilages can already be observed. Ossification in the anlage of the vomer is present in the 10½ day embryo by which time the vomerine cartilages are chondrified. In *Pyromelana* the vomerine cartilages, from the time they make their first appearance are closely applied to the dorsal laminae of the palatine and the vomer and never have any connexion with the trabecular bar. In *Anas* their mesenchymatous anlagen are closely attached to the ventro-lateral edges of the trabecular bar from which they are later freed; it is only after their chondrification that the vomer extends beneath them.

As far as the position of the earliest anlagen is concerned the development of the vomerine cartilages in *Anas* corresponds to that of the paraseptal cartilages in the lizard *Mabuia capensis*. In a series of *Mabuia* in this Institute the paraseptals chondrify in two strips of mesenchymatous tissue attached to the ventro-lateral edges of the base of the septum nasi. In the rabbit (DE BEER and WOODGER, 1930) the paraseptals also develop close to the ventral edge of the septum. It thus seems logical to regard the vomerine cartilages of *Anas* as homologous with reptilian and mammalian paraseptals. The manner of development of the

vomerine cartilages in *Pyromelana,* although differing from that in *Anas* and *Mabuia* in that they have no connexion with the ventral edge of the nasal septum in early stages, is not a very serious objection against regarding them as homologous with paraseptal cartilages. The condition in *Pyromelana* where the vomerine cartilages chondrify in the mesenchymatous tissue surrounding the developing vomer and not in separate mesenchymatous anlagen, as in *Anas,* is also found in *Mabuia,* where the paraseptal and vomer of each side develop in the same strip of mesenchymatous tissue attached to the lateral surface of the ventral edge of the septum.

W. K. Parker's observations on the vomerine cartilages in *Linota cannabina* (1876) and *Corvus frugilegus* (1875) suggest another possible explanation for the nature of these structures in *Pyromelana.* In the two forms mentioned he described the vomerine moities as developing as "endostoses" in the vomerine cartilages. It is thus possible that the vomerine cartilages in *Pyromelana* are merely parts of the vomer having a cartilaginous phase intercalated in their development, and that in their relations to the vomer they merely simulate paraseptals cartilages. This phenomenon, where part of a membrane bone passes through a cartilaginous phase is not uncommon and will be discussed in connexion with the cartilage associated with the palatine.

As can be inferred from the discussion above it is not intended to give a definite opinion on the homology of the Parkerian "vomerine cartilages" at this stage. A comparative investigation of the development of the vomer, the paraseptal cartilages and the vomerine cartilages in reptiles, birds and mammals, with special attention to the histogenesis of these structures is necessary before any definite conclusions can be made. Until such time it would be better to retain W. K. Parker's non-committal term "vomerine cartilages" for these structures in birds, on condition that the term is used in a purely descriptive sense.

G *The Visceral Arches*

The pars quadrata and Meckel's cartilage develop from one anlage, and it is only in the older embryos that active articulation between them is possible. The first part of Meckel's cartilage to develop is its retro-articular process. In the youngest stage the dorsal end of the pars quadrata is already directed antero-medially as an orbital process. A quadratopolar commissure develops between the medial surface of the orbital process and the antero-ventral corner of the polar cartilages. The first indication of this commissure is a procartilaginous protuberance on the medial surface of the pr. orbitalis a short distance behind its anterior tip. When the quadratopolar commissure becomes discontinuous owing to resorption of intercellular substance at a point some distance lateral to the point where the palatine nerve passes anteriorly below it, the ventral part

of the commissure persists for a short time as a medio-dorsally directed process on the inner surface of the pr. orbitalis. It is at this stage syndesmotically connected to the distal tip of the pr. basipterygoideus, which chondrifies in the dorsal part of the quadratopolar commissure. The process formed by the ventral part of the quadratopolar commissure is undoubtedly the basal process of the pars quadrata. The basal articulation in *Pyromelana* thus lies within the mandibular arch.

The basal process is of transitory nature and no trace of it is left in the older embryos. In the pigeon too, (FILATOFF, 1906) the ventral part of the quadratopolar commissure (columella of Filatoff) disappears.

The presence of a quadratopolar commissure makes it possible to establish the anterior border of the pars quadrata. As the basal process which develops from the ventral part of the quadratopolar commissure is a process of the pars quadrata, the pr. orbitalis must also be regarded as part of it and cannot thus be homologous with the pr. pterygoideus (part palatina) of the palatoquadrate of reptiles as SONIES (1907) maintained. Only the small portion of its anterior tip in front of the pr. basalis can be regarded as pars palatina. LANG (1954) regarded a cartilaginous plate associated with the palatine in *Passer* as a rudiment of the palatoquadrate, (probably of the pars palatina). An investigation of the youngest embryos of *Pyromelana* produced no evidence in favour of this viewpoint. At no stage during the development, could any connexion between the pars quadrata and the anlage of the cartilage associated with the palatine be observed.

The pr. oticus develops after the pr. orbitalis, as it also does in *Tinnunculus* (SUSCHKIN, 1899). Its medial head articulates with a facet formed by the otic capsule and the anterior edge of the metotic cartilage, and its lateral head articulates with the ventral edge of the squamosal. The facet on the squamosal is lined with cartilage developing in continuity with the lateral edge of the orbitocapsular commissure.

In the youngest stage the procartilaginous medial end of the anlage of the columella auris is fused to the procartilaginous floor of the cochlear portion of the otic capsule. In subsequent stages the medial tip chondrifies and enlarges to form the footplate of the columella auris. The footplate pushes into the capsular wall in which the intercellular substance is resorbed at this spot. No part of the capsule wall is included in the footplate, as SUSCHKIN (1899) maintained. The fenestra ovalis is formed around the footplate by resorption of the capsular wall.

In the older stages the pr. infracolumellaris is a long thin structure and apparently has no stylohyal fused to its distal tip. In fact, no trace of a stylohyal could be found in *Pyromelana*. FRANK (1954) described a similar condition in the ostrich. In *Sphenicus* (CROMPTON, 1953) a stylohyal is present but is not fused to the pr. infracolumellaris. In *Tinnunculus* (SUSCHKIN, 1899) the stylo-

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hyal forms the distal part of the pr. infracolumellaris ("pr. infrastapedialis"). The pr. supracolumellaris chondrifies in a postero-dorsally directed mesenchymatous protuberance of the extracolumellar part of the columella auris.

When intercellular substance is deposited in the anlage a small area is left devoid of any deposit. This unchondrified area becomes the "foramen of Huxley". A minute bloodvessel passes through the foramen. In stage IV a mesenchymatous band appears between the tip of the pr. supracolumellaris and the ventral surface of the metotic cartilage. In subsequent stages the pr. supracolumellaris increases in length by propressive deposition of intercellular substance in this band. In stage VIII this mesenchymatous band has completely disappeared.

The median copula is a product of fusion of two centres of chondrification (copula I and copula II) as in *Tinnunculus* (Suschkin, 1899), *Spheniscus* (Crompton, 1953) and *Struthio* (Frank, 1954). Copula I bears a mesenchymatous processus lingualis in the early stages which later chondrifies in continuity with it. Crompton (1953) maintained that in the young ontogenetic stages of the penguin a connexion exists between the hyoid blasteme and copula I. No such connexion is found in *Pyromelana*. In stage II however a posterolaterally directed process (lateral wing of the copula, Kallius, 1905) is present on the lateral surface of copula I. A similar process was described by Crompton in *Spheniscus*. Crompton did not definitely state that this process is a remnant of the earlier connexion between copula I and the hyoid blasteme but it is undoubtedly the case.

The epibranchial and the ceratobranchial develop as separate areas of chondrification within a single mesenchymatous anlage. Later they become fused by the deposition of intercellular substance in the mesenchymatous tissue connecting them. In the adult both elements are ossified, and it is not possible to determine the line of demarcation between them.

The paraglossals chondrify late during embryonic life and remain apart, as in the ostrich (Frank, 1954). The only connexion between them is a stout band of connective tissue in the region where their medial surfaces articulate with copula I.

H *Cartilage associated with the Os Palatinum*

This cartilage appears along the lateral edge of the posterior part of the palatine. When the bird is ready to leave the nest, the above mentioned cartilage still shows no sign of ossification. In the adult it is actually ossified and indistinguishably fused to the main bar of the os investitiens. Examination of earlier stages showed that this structure chondrifies in a portion of the same anlage in which the os investitiens ossifies. When the rod-shaped mesenchymatous anlage of the main bar of the palatine can be observed for the first time

the anlage of the cartilaginous structure is already present as a latero-ventrally directed flange of the posterior part of the anlage of the palatine. The cells in the anlage of the main bar of the palatine are more densely packed than in the flange, and in this part the density diminishes towards its lateral edge so that the flange appears to be an outgrowth of the main bar of the palatine. In older embryos the tissue of the main bar becomes more dense, and finally osteogenesis sets in in this region. It is only after the main bar of the palatine has assumed its definite shape that chondrification of the still mesenchymatous flange takes place.

We thus have a condition in which in one continuous mesenchymatous anlage both bone and cartilage are formed exactly as in the case of the vomer and the vomerine cartilages. The problem arising out of this state of affairs whether the bone resulting from the ossification of this cartilage must be considered as a part of the dermatocranium or as an ossification of the chondrocranium. W. K. PARKER (1876) regarded it as a transpalatine.

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In the light of the classic histogenetic criterion for distinguishing between ossa substitientia and ossa investitientia as applied by GAUPP (1906) and FUCHS (1922), it is an os substitiens. DE VILLIERS (1925 and 1926) regarded the ontogeny of the structures as the only valid criterion, maintaining that cartilage bone is always a derivative of the sclerotome while membrane bones have their origin in the cutis layer (Hautfasernblatt). Both Gaupp and de Villiers were aware of the circumstance that cartilage (so-called neomorphs) sometimes appear in the development of membrane bones. GAUPP (1906) considered it necessary to recognize more than these two categories in order to inclute these special structures of mixed histogenesis. He stated that the elements of the two categories develop in different localities (later to be clearly defined by DE VILLIERS (1925 and 1926) as the mesenchyme of the sclerotome and the cutis layer), and that each has its own independent development but that during the course of the development one type has acquired characteristics of the other.

After FUCHS (1926) had shown that the cartilage appearing in connexion with the human clavicle is not a neomorph but the remains of the procoracoid of lower vertebrates, DE VILLIERS (1926) was confident that further research on the ontogenesis of these neomorphs will reveal their true homology and will uphold the original (histogenetic) distinction between cartilage and membrane bones.

It seems, however, that the histogenesis of a bone is not always a sure guide of its origin. The centra of the vertebra in teleost fishes (REMANE, 1936) have a histogenesis typical of that of membrane bones, while these structures are undoubtedly of sclerotomic origin. On the other hand, the appearance of cartilage is a common phenomenon in the development of the dermatocranium of mammals where pieces develop in close association with certain dermal bones, and in places where it is difficult to regard them as persisting parts of the

chondrocranium of the mammalian ancestors. Interesting in this connexion is the work of KOLLER (1896) referred to by GAUPP (1901). Koller observed that in the healing of fractures of undoubted membrane bones e.g. the jugale, a cartilaginous callus appears.

It would thus seem that the property of cells to differentiate into either chondroblasts or osteoblasts is not confined to those cells of sclerotomic origin only but is also a property of cells originating in the cutis layer. As a general rule cells of sclerotomic origin differentiate into both chondroblasts and osteoblasts (except in the case of the centra of the vertebra of teleost fiches), while cells from the cutis layer differentiate into osteoblasts and only rarely into chondroblasts. The conclusion which we come to is that the ontogenetic criterion is the only valid one for determining whether a skeletal structure is part of the dermal skeleton of not, and that the histogenesis of a structure can sometimes be misleading. The skeletal structures resulting from the ossification of the cartilage associated with the palatine and the vomerine cartilages in *Pyromelana* can therefore be regarded as parts of the dermal bones to which they become fused subsequent to their ossification, having a cartilaginous phase intercalated in their development.

It is, however, equally reasonable to maintain that the chondroblasts may not have originated from the cutis layer and may have migrated to the spot where they will eventually give rise to cartilage. Micro-chemical tests are not yet accurate enough to aid in the solving of the problem.

Cartilage occurring in the development of membrane bones has been referred to in the literature as secondary cartilage, and according to DE BEER (1937) it shows remarkable resemblance to hypertrophic cartilage which develops in those parts of the primary cartilage of the embryonic skeleton in which ossification is about to take place. This observation was probably made on material in which such cartilages were about to be replaced by bone. FUCHS (1909) described a nodule of cartilage developing in the maxillary of the cat as typically hyaline. In *Pyromelana*, the histogenesis of the cartilage associated with the palatine proceeds exactly in the same manner as that of the cartilage of the chondrocranium. In birds ready to leave the nest the cartilage still has the appearance of normal hyaline cartilage with no signs of hypertrophy of the chondrocytes.

"Neomorphic" cartilage associated with the histogenesis of some ossa investitientia is an extremely difficult morphological problem. The matter is tentatively mentioned here, but it cannot be pretended that for *Pyromelana* the problem has been solved.

I *The Ossa Palatomaxillaria*

These bones are the ossifications of the cartilagines palatomaxillares which chondrify in the tendons of the m. adductor mandibulae externus. Ossification

only takes place after the young bird has left the nest; immediately prior to this the cartilages show no signs of ossification. In the adult they are ossified and synostotically connected to the dorsal edge of the supra-angular. The pneumatic cavity of the lower jaw extends into the posterior ossicle. The anterior ossicle contains small interconnected marrow cavities. Their cartilaginous predecessors are typically hyaline cartilage. Where the tendons attach to the cartilages, chondrocytes and intercellular substance spread out into the tendons for some distance. WEIDENREICH (1923) described a similar condition in the attachment of the Achilles tendon to a protuberance of the calcaneus of man. He referred to the tissue between the tendon proper and the hyaline protuberance as "Faserknorpel", and maintained that it ossified and was resorbed together with the calcified cartilage of the protuberance. The latter was then sustituted by lamellate bone.

Unfortunately no stages of fledglings were available so that it was not possible to study the process of ossification in these cartilages. Apparently it follows the same course as in the material described by Weidenreich. In the adult the ossicles consist of typical lamellate bone identical with that of the supra-angular to which they are fused. They are thus not "Faserknochen" (WEIDENREICH, 1923).

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FÜRBRINGER (1888) and LEBEDINSKY (1921) remarked that ossification in tendons was a common phenomenon among birds in old age and that their occurrence in various parts of the body was of no morphological interest. There are, however, skeletal structures which occur at definite localities, and because they occur only in certain genera or families, they are of taxonomic interest. Structures of this type were described by FÜRBRINGER (1888) as "Sesamgebilde" or "Sesamkörper". The ossa palatomaxillaria of *Pyromelana* undoubtedly belong to this category as they are constant as far as their number and topography are concerned. The ossicles along the dorsal edge of the supra-angular in birds were first described by NITZSCH (1811) as "Mundwinkelbeine" or ossa palatomaxillaria. Magnus referred to them in 1870. KALLIUS (1905) found an isolated cartilaginous nodule near the supra-angular (his coronoid) in the duck and sparrow. DE BEER (1937) observed this nodule in the duck and called it the "coronoid cartilage". Unfortunately Kallius and de Beer did not ascertain its ultimate fate, but it is undoubtedly the cartilaginous predecessor of the palatomaxillary bone.

It does not seem possible that the cartilaginous predecessors of the ossa palatomaxillaria in birds can in any way be regarded as homologous with the "coronoid cartilage" described by W. K. PARKER (1883) in the crocodile. This structure is situated on the medial side of the coronoid bone and articulates with the "pterygoid cartilage" on the dorsal surface of the pterygoid. As the use of the term "coronoid cartilage" in the crocodile and bird implies homology, it is better to retain Parker's term for the crocodilian structures and to refer to the car-

tilaginous predecessors of the avian ossa palatomaxillaria as cartilagines palatomaxillares.

II SUMMARY

(1) The anterior tip of the chorda does not pierce the anterior surface of the acrochordal.
(2) A fenestra basicranialis develops by resorption.
(3) In the young embryos the anterior hypoglossal root has more than one foramen.
(4) Three procartilaginous cranial ribs are present in the youngest stage.
(5) The metotic cartilage develops from two separate anlagen.
(6) The posterior and the lateral edges, and the lateral part of the anterior edge of the metotic cartilage fuse with the otic capsule.
(7) The ventral surface of the metotic cartilage has a plate-like outgrowth.
(8) The vagus leaves the cranial cavity through its own foramen.
(9) The acustic foramina and the foramen for the endolymphatic duct are remnants of the dorsal part of the cochleo-canalicular fissure.
(10) The foramen perilymphaticum and the foramen ovale arise as a result of resorption of the capsular wall.
(11) The facial is included in a canal in the anterior wall of the otic capsule.
(12) In the youngest embryo the polar cartilage can be distinguished histologically from the trabecula.
(13) The orbitonasal septum develops as an upgrowth of the trabecula communis.
(14) A procartilaginous quadratopolar commissure is present in the young embryo.
(15) The pr. basipterygoideus chondrifies in the dorsal part of the quadratopolar commissure.
(16) An infrapolar process is absent.
(17) A protuberance of the basal plate participates in the formation of the infracarotid commissure.
(18) The orbital cartilage is never complete.
(19) The anterior part of the orbital cartilage fuses to the dorsal part of the interorbital septum and temporarily encloses the olfactory nerve in a canal.
(20) The anterior part of the orbital cartilage is completely resorbed in the nestling.
(21) An orbitocapsular commissure develops between the postorbial part of the orbital cartilage and the otic capsule.
(22) A pila antotica spuria is present.

(23) The nasal capsule—with the possible exception of the planum antorbitale develops from the dorsal edge of the nasal septum.
(24) A paranasal cartilage is absent.
(25) The fenestra narina is completely surrounded by cartilage.
(26) A solum nasi is present in the anterior region of the nasal capsule.
(27) An accessory atrioturbinal cartilage is present.
(28) The latero-ventral corner of the planum antorbitale is pierced by the maxillary branch of the trigeminal.
(29) A pair of elongated vomerine cartilages are present.
(30) A well-developed prenasal process is present, but is completely resorbed in the nestling.
(31) The ventral edge of the septum nasi is thickened.
(32) This thickened part (trabecular bar) is completely resorbed in the nestling.
(33) The first part of Meckel's cartilage to develop is the pr. retroarticularis.
(34) A short pr. basalis is present in the young embryo.
(35) The pr. orbitalis is part of the pars quadrata of the palatoquadrate.
(36) The medial end of the columella auris is fused to the capsular wall in the youngest stage.
(37) The wall of the otic capsule does not contribute towards the formation of the foot-plate of the stapes.
(38) No stylohal is included in the pr. infracolumellaris.
(39) The base of the pr. supracolumellaris is pierced by Huxley's foramen.
(40) The paraglossal cartilages never coalesce.
(41) The lateral part of the palatine bone apparently passes through a cartilaginous phase during ontogeny.
(42) Two cartilagines palatomaxillares chondrify in the tendons of the m. adductor mandibulae externus.
(43) A cartilaginous nodule develops in the tendon of the m. pseudotemporalis superficialis and fuses with the dorsal surface of Meckel's cartilage.

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IV ALPHABETICAL LIST OF ABBREVIATIONS USED IN THE FIGURES

ACC. ATR. CAR. Accessory atrioturbinal cartilage
A.C.R. Area where cartilage has been resorbed
ACR. CAR. Acrochordal cartilage
A.L.R.S.T. Apertura lateralis recessus scaele tympani
A.M.R.S.T. Apertura medialis recessus scalae tympani
ANL. I. BRAN. ARC. Anlage of the first branchial arch
ANL. COP. Anlage of the copula
ANL. PAL. Anlage of the palatinum
ANL. POS. ORB. CAR. Anlage of the posterior part of the orbital cartilage
ANL. PROC. EXT. & SUP.COL. Anlage of the processus extracolumellaris and the processus supracolumellaris
ANL. PROC. INF. COL. Anlage of the processus infracolumellaris
ANL. PT. Anlage of the pterygoid
ANL. STAP. Anlage of the stapedial part of the columella auris
ANL. TEC. SYN. Anlage of the tectum synoticum
ANL. TRAB. Anlage of the trabecula
ANT. ORB. CAR. Anterior part of the orbital cartilage
ATR. TUR. CAR. Atrioturbinal cartilage
BAS. PL. Basal plate
B.M.T. Band of mesenchymatous tissue
BR. Brain
CAR. A.W. PAL. Cartilage associated with the palatine
CAR. N. Cartilaginous nodule
CAR. N.M.P.S. Cartilaginous nodule in the musculus pseudotemporalis superficialis
CAR. PALA. MAX. Cartilagines palatomaxillares
CER. BRAN. Ceratobranchial
C.M.T.P.A. Connexion between the maxilloturbinal cartilage and the planum antorbitale
COCH.-CAN. FIS. Cochleo-canalicular fissure
COP. I. First copula
COP. II. Second copula
C.P.N.P.A. Connexion between the paries nasi and the planum antorbitale
C. QUA. JU. Concavity lodging the posterior end of the quadratojugal
CRA. FAC. FEN. Craniofacial fenestra
CRAN. RIBS. Cranial ribs
CUP. ANT. Cupola anterior
D.D.L. NAS. GL. Dorsal duct of the lateral nasal gland
D.O. OLF. CA. Dorsal orifice of the olfactory canal
D.O. TROC. CA. Dorsal orifice of the trochlear canal
DUC. NAS. LAC. Ductus nasolacrimalis
EPI. BRAN. Epibranchial
FEN. BAS. POS. Fenestra basicranialis posterior
FEN. NAR. Fenestra narina
FEN. SEP. Fenestra septalis
FEN. SEP. NAS. Fenestra septi nasi
FIS. MET. Fissura metotica
FOR. ABD. Foramen for the abducent nerve
FOR. CAR. Foramen caroticum
FOR. END. Foramen endolymphaticum
FOR. FAC. Foramen for the facial nerve
FOR. HYPOP. Foramen hypophyseos
FOR. ACU. Foramina acustica
FOR. OC. Foramen for the oculomotor nerve
FOR. OLF. AD. Foramen olfactorium advehens
FOR. OLF. EV. Foramen olfactorium evehens
FOR. OPHT. Foramen for the ophthalmic artery
FOR. OVAL. Foramen ovale
FOR. PERI. Foramen perilymphaticum
FOR. PROF. Foramen for the profundus nerve
FOR. PRO. SPU. Foramen prooticum spurium
FOR. R. MAX. Foramen for the ramus maxillaris of the trigeminal nerve

FOR. VAG.-ACC. Foramen for the vagus and accessory nerves
FR. Frontal
G. GEN. Ganglion geniculatum
G.L.N.C. Groove lodging the notochord
HYP. FOR. Hypoglossal foramina
HYP. PIT. Hypophysial pit
HUX. FOR. Huxley's foramen
INC. CAR. Incisura carotica
INC. PRO. Incisura prootica
INF. CAR. COM. Infracarotid commissure
LAT. ANL. MET. CAR. Lateral anlage of the metotic cartilage
L.H. PROC. OTIC. Lateral head of the processus oticus
L.S.L. ATR. Laterally directed secondary lamella of the atrioturbinal cartilage
MAX. Maxillary
MAX. TUR. CAR. Maxilloturbinal cartilage
MEA. ACUS. EXT. Meatus acusticus externus
MEC. CAR. Meckel's cartilage
MED. ANL. MET. CAR. Medial anlage of the metotic cartilage
M.E.P.N. & A.T. Medial extension of the paries nasi and the atrioturbinal cartilage
MET. CAR. Metotic cartilage
M.H. PROC. OTIC. Medial head of the processus oticus
MID. TUR. CAR. Middle turbinal cartilage
M. PROC. Mesenchymatous process
M.S.L. ATR. Medially directed secondary lamella of the atrioturbinal cartilage
NAS. Nasal
N.C. Notochord
OLF. Olfactory nerve
OLF. CA. Olfactory canal
OCC. CON. Occipital condyle
ORB. CAP. COM. Orbitocapsular commissure
ORB. CAP. PROC. Orbitocapsular process
OTIC CAP. Otic capsule
O.V.D.L. NAS. GL. Orifice of the ventral duct of the lateral nasal gland
PAL. Palatine
PARA. GLOS. CAR. Paraglossal cartilage
PARA. ROS. Parasphenoid rostrum
PAR. NAS. Paries nasi
PARS. CAN. Pars canalicularis
PARS COCH. Pars cochlearis
PARS. LAT. OCC. Pars lateralis of the occipital region
P. BAS. PL. Protuberance of the basal plate
PIL. ANT. Pila antotica
PIL. ANT. SPU. Pila antotica spuria
PIL. OCC. Pila occipitalis
PLAN. ANT. Planum antorbitale
P.L. ATR. Primary lamella of the atrioturbinal cartilage
P. PERIC. Persisting perichondrium
POL. CAR. Polar cartilage
POS. ORB. CAR. Posterior part of the orbital cartilage
PR. Praefrontal
PRE. NAS. Prenasal process
PROC. ALV. O. PR. Processus alveolaris ossis praemaxillaris
PROC. ANG. Processus angularis medialis
PROC. BAS. Processus basalis
PROC. BAS. PTE. Processus basipterygoideus
PROC. ETH. PAL. Processus ethmopalatinus
PROC. EXT. COL. Processus extracolumellaris
PROC. FR. O. NAS. Processus frontalis ossis nasalis
PROC. FR. O. PR. Processus frontalis ossis praemaxillaris
PROC. INF. COL. Processus infracolumellaris
PROC. LING. Processus lingualis
PROC. MAX. O. NAS. Processus maxillaris ossis nasalis
PROC. MAX. PAL. Processus maxillopalatinus
PROC. M.E.P.N. & A.T. Process of the medial extension of the paries nasi and the atrioturbinal cartilage
PROC. MET. CAR. Process of the metotic cartilage
PROC. ORB. Processus orbitalis
PROC. OTIC Processus oticus
PROC. PAL. O. PR. Processus palatalis ossis praemaxillaris

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PROC. PR. O. NAS. Processus praemaxillaris ossis nasalis
PROC. RET. Processus retroarticularis
PROC. SUP. COL. Processus supracolumellaris
PROC. PTE. Processus pterygoideus
PROC. ZYG. O. MAX. Processus zygomaticus ossis maxillaris
PROM. SEM. LAT. Prominentia semicircularis lateralis
PRO. PROC. Prootic process
QUAD. POL. COM. Quadratopolar commissure
QUA. JU. Quadratojugal
PTE. Pterygoid
REC. SCA. TYM. Recessus scale tympani
RE. PRE. NAS. Remnant of the prenasal process
RE. TRAB. BAR. Remnant of the trabecular bar
R. INT. SEP. Ridge of the interorbital septum
R.L. NAS. Ramus lateralis nasi
R.M. NAS. Ramus medialis nasi
R. PAL. Ramus palatinus
SCL. CAR. Sclerotic cartilage
S.C.T. Strand of connective tissue
SEP. INT. Septum interorbitale
SEP. NAS. Septum nasi
S.L. MAX. TUR. CAR. Secondary lamella of the maxilloturbinal cartilage
SOL. NAS. Solum nasi
SQUA. Squamosal
STAP. Stapedial part of the columella auris
SUP. ORB. CAR. Supraorbital part of the orbital cartilage
SUP. POL. CAR. Suprapolar cartilage
TEC. NAS. Tectum nasi
TEC. SYN. Tectum synoticum
T.L. ATR. Tertiary lamella of the atrioturbinal cartilage
T.M.T. Tract of mesenchymatous tissue
TRAB. BAR. Trabecular bar
TRAB. COM. Trabecula communis
V.D.L. NAS. GL. Ventral duct of the lateral nasal gland
VOM. Vomer
VOM. CAR. Vomerine cartilage
V.O. TROC. CA. Ventral orifice of the trochlear canal

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ON THE DORSAL GANGLION CELLS OF THE SPINAL CORD IN LAMPREYS*

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1. INTRODUCTION

The position of the so-called dorsal cells ("Hinterzellen" REISSNER 1860) within the gray matter of the spinal cord in cyclostomes as well as the main features of these cells have been known for a long time (MÜLLER 1839, OWSJANNIKOW 1854, REISSNER 1860. The early literature is reviewed by STUDNIČKA 1895, see also WHITING 1948). As a rule these cells have two large cells processes and it has been repeatedly observed that one of these large processes often joins the fibres of a dorsal root (see FREUD 1878, STUDNIČKA 1895, BECCARI 1909). For this reason, as it seems, most authors have regarded the dorsal cells as sensory (see IHLE etc. 1927, ARIENS KAPPERS 1934, ARIENS KAPPERS, HUBER & CROSBY 1936, HORST 1934, SCHULTZ, BERKOWITZ & PEASE 1956, SCHARF 1958). WHITING (1948, 1955) studied the dorsal cells of the spinal cord of "proammocoete" stages of lampreys and found cell processes from these cells which terminated in the skin. Consequently these cells were considered to be somatic sensory and, in addition, WHITING considered the dorsal cells to be homologous with the Rohon-Beard cells of lower gnathostomes. On the other hand, although no distinct indication of a motor nature of these dorsal cells in

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lampreys was given, STUDNIČKA pointed out the possibility that they might be somatic motor and, further, denied any homology with the Rohon-Beard cells of gnathostomes (selachians). According to TRETJAKOFF (1909) the dorsal cells and their cell processes were altogether intra-medullar and consequently without contact with the fibres of the dorsal roots. He considered them to be association neurons (see also PIETSCHMANN 1933).

In a recent paper dealing with the structure of the peripheral autonomic nervous system of the trunk region in lampreys the present author mentioned the fact that it had been possible to trace peripheral autonomic fibres centrally through the dorsal spinal roots to their origin in dorsal cells within the spinal cord (JOHNELS 1956). The present study is a consequense of this observation which gives a new idea of the nature of dorsal cells in the spinal cord of lampreys.

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2. MATERIAL AND METHOD

It has been stated by previous authors that the dorsal cells are particularly difficult to stain with ordinary silver impregnation methods as well as with methylene blue techniques (KOLMER 1905, TRETJAKOFF 1909). However, it has been possible in the present work to stain very selectively the fibres of these cells within as well as outside the spinal cord and to impregnate the cell bodies tolerably well by means of a modification of the Bodian protargol method (JOHNELS 1955). The cell bodies also have been studied with a silver nitrate impregnation method elaborated by PALMGREN. The peripheral and intra-medullar distribution of the cell processes have been studied on sectioned material of larval (*Lampetra planeri*) and adult lampreys (*Lampetra planeri* and *L. fluviatilis*). As stated previously (see JOHNELS 1956) many of the visceral fibres of dorsal roots are particularly thick and this quality has made it possible to trace them from the periphery into the spinal cord and within the spinal cord to their origin from a dorsal cell. In special cases the method of graphic reconstruction has been adopted.

3. THE CELL BODIES OF DORSAL CELLS

The dorsal cells are situated in roughly four rows within the spinal cord, two on each side of the median plane (see ARIENS KAPPERS, HUBER & CROSBY 1936). The cells are located close to the ventral surface of the fibrous tissue of the dorsal white matter. On each side one row of cells is close to the ependyma of the central canal. The other two rows are more laterally disposed in the gray matter and are situated dorsally to the dorsal giant Müllerian fibres (see

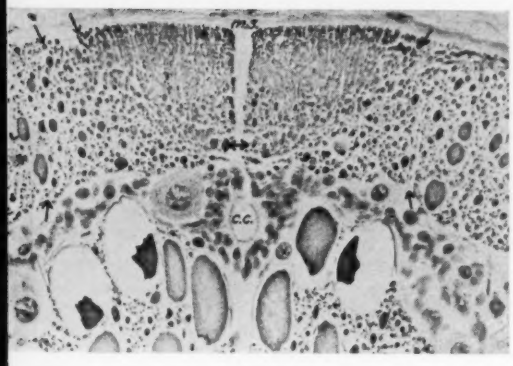

Fig. 1

Fig. 2

Fig. 1. Microphotograph of transverse section of spinal cord of *Lampetra planeri*. 300 ×. Smooth dorsal cell to the left of the central canal (c.c.). Dorsally to the central canal the median septum (m.s.) between the dorsal funiculi. The limits between the dorsal, the dorso-lateral and lateral funiculi indicated by arrows. In upper right corner obliquely cut fibres at the base of a dorsal root. Three of the fibres of Müller have collapsed. Bodian method.

Fig. 2. Transverse section of spinal cord. *Lampetra planeri*. 300 ×. To the right of the central canal a smooth dorsal cell and to the left rough dorsal cell. The dark spot on the dorsal border of the smooth cell i a fibre belonging to an adjacent dorsal cell. A number of neurofibrils are visible in this fibre. The rough dorsal cell is traversed close to the dorsal surface by a fibre belonging to another dorsal cell. m.s. = median septum. Bodian method.

Figs. 1 and 2). The white matter dorsally of the dorsal cells is divided into a dorsal funiculus bordering on the median septum and a dorso-lateral funiculus, the fibres of which are in relation to the dorsal roots and, as will be discussed below, to the dorsal cells (see Fig. 1).

The shape of the cell bodies of dorsal cells of lampreys has been described and pictured by STUDNIČKA (1895), TRETJAKOFF (1909) and BECCARI (1909). With very rare exceptions there are two main cell processes on each cell, and, according to the different ways in which these processes emerge from the cell body, the shape of the cell i affected. BECCARI distinguished between four types of dorsal cells. Of these types three were regarded as bipolar with minor differences as regards the mutual size and position of the two main cell processes, and one type was represented by cells with a more or less definite tendency towards being unipolar as regards the main cell processes. The basis of this systematization was, thus, variations in the morphological relations between the two large cell processes and between these and the cell body. However, STUDNIČKA and BECCARI have observed that in addition to these large cell processes there are also many small ones situated on the surface of the cell body of dorsal cells. This is evident also from their figures. For this reason STUDNIČKA considered the dorsal cells to be multipolar and KOLMER (1905) was of the same opinion.

As mentioned previously there are difficulties in staining the cell bodies of

dorsal cells (STUDNIČKA, KOLMER, BECCARI, TRETJAKOFF) and this is particularly true as regards these small cell processes on the bodies. In consequense they may easily be overlooked in the preparations. In the present material the same difficulty has arisen but in general the existence of these small cell processes is indicated even if they are not stained. The surface of the cells appears uneven or rough because of the small extensions of the cell surface at the base of these small cell processes (see Fig. 2). However, among STUDNIČKA's as well as BECCARI's pictures of dorsal cells there is one example of a cell with no small cell processes and a completely smooth outline. This has not given rise to any particular comment in their texts. TRETJAKOFF (1909) noticed that some of the dorsal cells were distinctly "bipolar" while others have small cell processes on the cell body. This is of considerable interest in connection with the following observation.

In the present material many thick autonomic fibres of the trunk region (JOHNELS 1956) have been traced into the spinal cord and in *all cases* the origin of the fibres has been in a dorsal cell *with a completely smooth outline* in which the small processes of the cell body are missing. This regular relation between thick autonomic fibres and such smooth dorsal cells strongly indicates that these cells represent a special type of dorsal cells, different from the other type (or types), represented by the cells with a rough surface which have been described above. These types of dorsal cells are subsequently termed the smooth type and the rough type.

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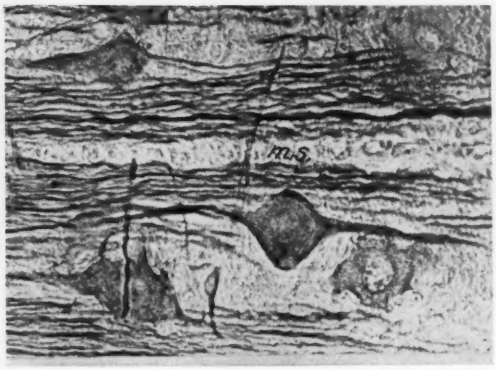
a) THE SMOOTH TYPE OF DORSAL CELL

The smooth type of dorsal cell is mostly very regular in shape. In transverse sections (Fig. 1, 2 and 5 c) it is almost circular or slightly ovoid (particularly in *Lampetra fluviatilis*) and in horizontal (Fig. 3) or sagittal (Fig. 4) sections it appears rounded or, more often, ovoid to spindle-shaped. In the sections one large cell process is seen to leave the cell body anteriorly and the other posteriorly (Figs. 3, 4). In some cases the cell body may be more convex on one, usually the ventral, side than on the other in which cases the cell bodies are more or less asymmetric. The stainability of the plasm of the smooth type is slightly higher than that of the rough type of dorsal cell. Often the staining reveals a multitude of neurofibrils in the plasm of the smooth dorsal cells. The neurofibrils seem to be arranged mainly longitudinally within the cell (Fig. 5 c) and gather to a bundle at each end where they run into the large cell processes (see Fig. 2). The nucleus is ovoid and regular in contour (Fig. 1). It contains one distinct nucleolus (Fig. 4).

In a few cases extremely delicate structures have been observed which may represent cell processes on the cell surface. The methods adopted in the present work do not allow a definite statement to the effect that there are no small cell



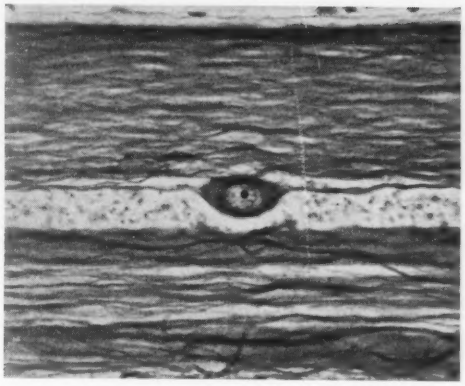


Fig. 3 Fig. 4

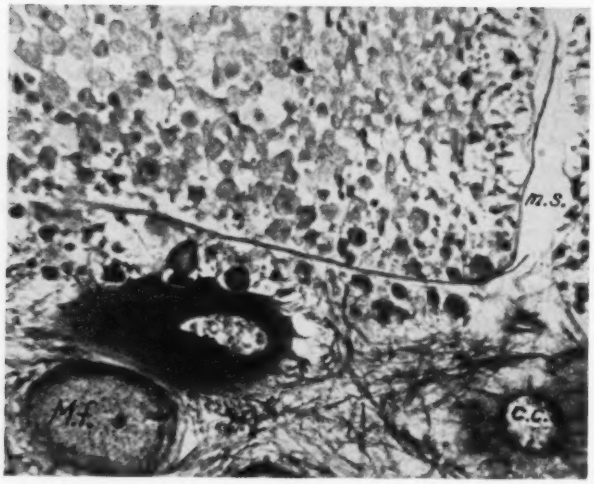
Fig. 3. Horizontal section of spinal cord of *Lampetra planeri*. 300 ×. One smooth dorsal cell and four rough cells among the fibre bundles. m.s. = median septum. Bodian method.

Fig. 4. Sagittal section of spinal cord. Larva of *Lampetra planeri*. 450 ×. One smooth dorsal cell in the gray matter with cell processes. Bodian method.

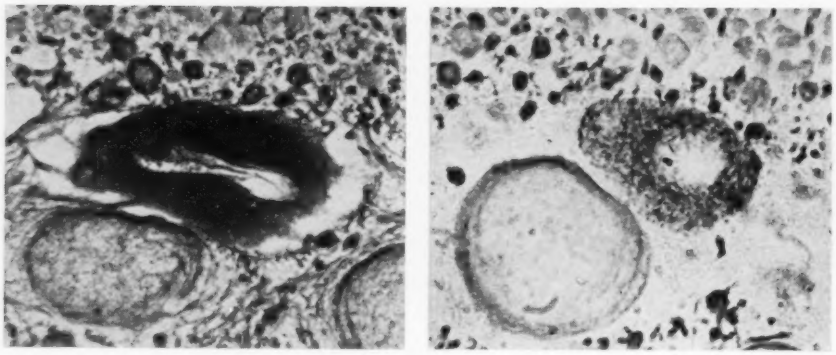
processes on the cellular surface of the smotth cells, although the possibility that such processes do exist appears very slight.

b) THE ROUGH TYPE OF DORSAL CELL

The rough type is much commoner than the smooth type (Fig. 3). As is evident from earlier descriptions in the literature (STUDNIČKA, BECCARI) the shape of this type of dorsal cell is variable, due mainly to the way in which the emergence of the main cell processes takes place. However, also the small cell processes may add to this polymorphism as they are often numerous and rather irregularly arranged on the surface of the cells (Figs. 2, 5 A, B). Often the main cell processes emerge from the anterior and posterior ends of these rough cells but in most cases the places of emergence are irregularyl arranged as is evident from BECCARI's descriptions. The shape of the nucleus is elongate and often irregular (particularly in *Lampetra fluviatilis*). That this was so in the case of some of the nuclei of dorsal cells was noticed by TRETJAKOFF (1909). The nuclear membrane is often folded in the large cells of *lampetra fluviatilis* (Fig. 5 B). There is one nucleolus. Inclusions in the cell plasm are common (particularly in *Lampetra fluviatilis*). There are preparations where neurofibrils are visible in the plasm of the rough cells, particularly in the vicinity of the base of the large cell processes. However, the arrangement of neurofibrils within the plasm of the cell body seems to be less regular and sparser than in the smooth cells. There seems to be no distinct indication in the shape of these rough dorsal cells as to whether they represent variations of a single kind of



A



B C

Fig. 5. From transverse sections of spinal cord of *Lampetra fluviatilis*. 500 ×. A. Rough cell. Note intra-medullar vascular branch (belonging to a smooth dorsal cell) situated dorsally to the cell and in the median septum. M.f. = fibre of Müller, m.s. = median septum, c.c. = central canal. B. Rough cell. Note nucleolus to the left in the nucleus. Longitudinal folds of nuclear membrane seen as dark lines in the nucleus. C. Smooth dorsal cell. The dotted appearance of the plasm is due to the intense impregnation of neurofibrils which are oriented longitudinally within the cell and, consequently, are cut transversally in this section. A. and B. Palmgren method, C. Bodian method.

nervous cell or whether—as assumed by BECCARI—they represent different kinds.

There are cases in which there may be difficulties in deciding whether a cell body belongs to the smooth type or the rough type. This is particularly so in material which is improperly fixed. If the cell bodies appear shrunken there may be artifacts imitating many true cell processes also on smooth cells. It

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seems likely that unsatisfactory fixing is the explanation of the fact that previous authors did not distinguish between the smooth and rough cells. This is indicated by the collapsed nature of the giant Müller's fibres in most of the figures given by previous authors. These elements are extremely susceptible to inaccurate fixing and may serve as an indicator of the nature of the fixing.

4. THE CELL PROCESSES OF THE DORSAL CELLS

The fact that one of the cell processes of a dorsal cell often joins the fibre bundle of a dorsal root has been observed repeatedly (KUTSCHIN in STIEDA 1866, FREUD 1878, STUDNIČKA 1895, BECCARI 1909). On the other hand KOLMER (1905) observed this in one single case only and TRETJAKOFF (1909) completely denied this, stating that the dorsal cells and their cell processes were completely intra-medullar.

a) THE CELL PROCESSES OF THE SMOOTH TYPE OF DORSAL CELL

The smooth type of dorsal cell only has the two main cell processes. Of these one is directed anteriorly and the other posteriorly within the spinal cord. As mentioned above it has turned out that always and without exception when a peripheral visceral fibre of the thick type has been successfully traced to its origin in the spinal cord this has been in a smooth dorsal cell. On the other hand when fibres have been traced the other way from this smooth type of cells and distally one of the two fibres has emerged—often at a considerable distance from the cell body—through a dorsal root in the shape of a thick visceral fibre. The large number of such observations seems to allow of the conclusion that the smooth dorsal ganglion cells are visceral in nature (JOHNELS 1956). The fact that the connection between fibres of the dorsal roots and the dorsal cells escaped TRETJAKOFF must be due to his methods, probably the dissecting technique.

Of the two cell processes of a smooth dorsal cell one is often distinctly thicker than the other. This thick fibre is the peripheral one which will leave the spinal cord through a dorsal root. This fibre runs for a shorter or longer distance longitudinally within the medial or dorsal portion of the dorso-lateral funiculus (see Figs. 1, 4, 6, 7 A) of the spinal cord from the cell body and to the level of the pertaining dorsal spinal root. Here, the fibre turns abruptly towards the spinal root emergence and leaves the medulla. The peripheral distribution of this type of fibre (see Fig. 6) has been described previously (JOHNELS 1956). The termination within the viscera indicates that this fibre type is viscero-motor, probably vaso-motor.

The other fibre emerging from a smooth dorsal cell runs in the opposite

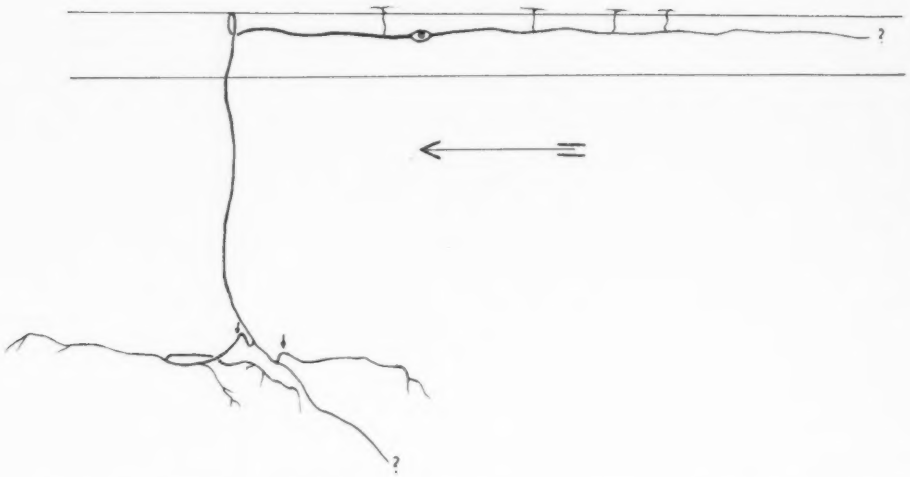

Fig. 6. Reconstruction of smooth dorsal cell with cell processes. *Lampetra planeri*. Left lateral view. 40 ×. The large arrow indicates the direction towards the head. Small arrows indicate the places where branches enter the horizontal connective tissue membrane ventrally to the left cardinal vein on their course towards the viscera (see JOHNELS 1956). Outline of spinal cord indicated. Undecided termination of fibres indicated by ? The undulations of the intra-medullary fibres indicate the position of dorsal cells situated ventrally of the fibre.

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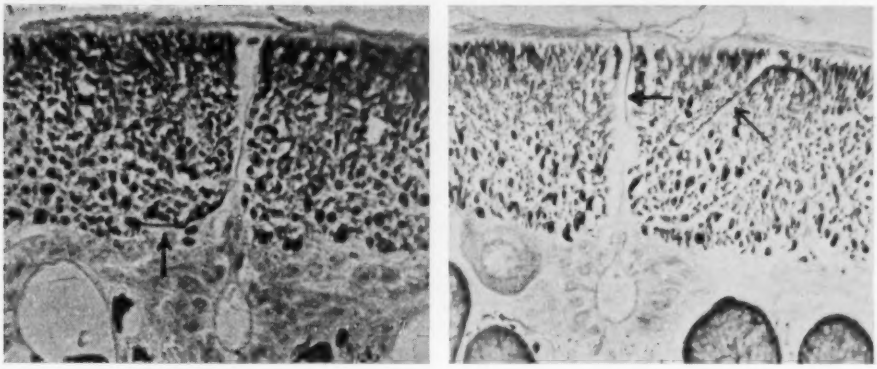
direction from the cell body in relation to the peripheral one (Figs. 4, 6). These fibres run in the ventro-lateral part of the dorso-lateral funiculus. Usually they gradually approach a more and more lateral position within the funiculus. These fibres—which are here termed the funicular fibres as distinguished from the peripheral ones—gradually become thinner and when the fibres become too thin their relations to neighbouring fibres become too confused to allow them to be safely traced in the sections. Consequently the extreme ends and terminations of these funicular fibres have not been observed.

However, a remarkable feature of these funicular fibres has been noted. From these longitudinal, funicular fibres several branches are given off in a medial direction towards the septum which separates the two dorsal funiculi in the median line dorsally to the central canal (Figs. 6, 7, see also Fig. 5 A). Mostly these branches run ventrally to the fibrous funiculi on the border of the gray matter mainly perpendiculary to the longitudinal axis of the spinal cord. In some cases these branches have their course through the fibrous tissue of the funiculi towards the median septum (Fig. 7 B). In the tissue of the median septum the branches turn dorsally and run out of the medulla penetrating the meninx primitiva. These septal fibres were noticed by TRETJAKOFF who stated that they belonged to fibres of dorsal roots but had no immediate relation to dorsal cells. As all the funicular and also a number of the peripheral fibres of



A B

Fig. 7. Transverse sections of spinal cord of *Lampetra planeri*. Bodian method. 450 ×. A. Vaso-motor branch given off from intra-medullar funicular branch of a smooth dorsal cell. Position indicated by an arrow. B. Unusual course of vaso-motor intra-medullary branch (indicated by arrows). The terminal part of the fibre in the picture is seen to penetrate the meninx primitiva.

smooth dorsal cells give off such branches the tissue of the median septum is richly supplied with these vertical fibres. The great number of these small nerve fibre branches is particularly striking in sagittal sections of the spinal cord (Fig. 8). Usually they are rather thin but in some cases thick fibres have been observed. Mostly they do not branch within the spinal cord. TRETJAKOFF regarded them as intra-medullar terminations.

As is well known the exterior surface of the spinal cord in lampreys is covered by a dense network of arterioles and capillaries (STERZI 1904, see also TRETJAKOFF 1909, KAPPERS, HUBER & CROSBY 1936). Outside the meninx primitiva of the spinal cord the small branches of the smooth dorsal cells which have been described above form a terminal system of very delicate fibres by repeated branching (Fig. 9). This gives a rich supply of fibres just outside the mentioned network of extra-medullar blood vessels. In this nerve net the thin terminal fibres have a definite tendency to run along the small blood vessels and capillaries.

It has been preciously demonstrated that the terminal branches of the peripheral visceral fibres of the smooth dorsal cells have distinct expansions along their course (JOHNELS 1956). This is also so in the case of the terminal fibres of the branches to the spinal cord blood vessels (Fig. 9). These expansions are very similar to small droplets. They do not stain positively with chrome alum haematoxylin-phyloxine according to GOMORI (1941) or paraldehyde fuchsin--pikroindigo carmine trioxyhaematein according to GABE (1953).

Such branches to the extra-medullary blood vessels are often present also on the peripheral fibres of the smooth dorsal cells (indicated in Fig. 6). This

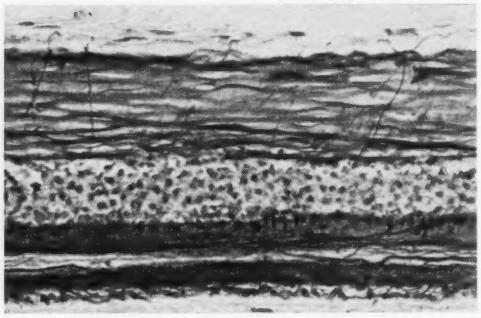


Fig. 8. Sagittal section through median septum of spinal cord. *Lampetra planeri*. 300 ×. The vertical fibres which cross the longitudinal bundles of fibres of the dorsal funiculus are the vertical parts of the intra-medullary branches to the vessels outside the meninx primitiva. Bodian method

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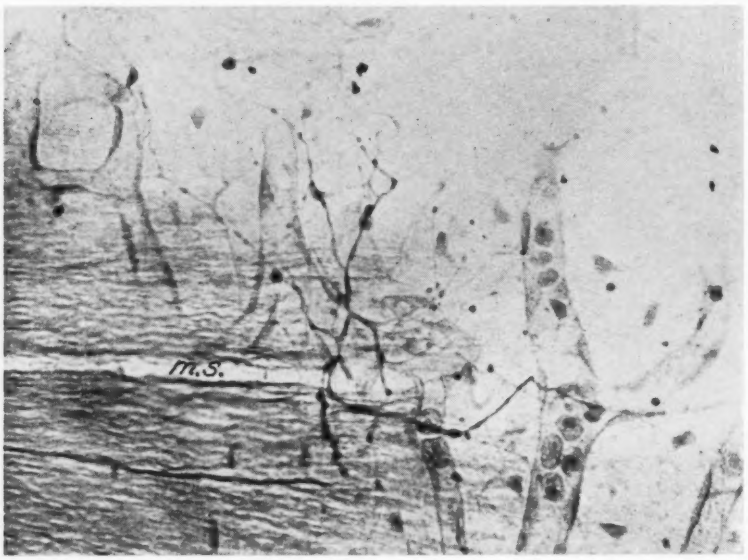

Fig. 9. Slightly oblique horizontal section of spinal cord. *Lampetra planeri*. 450 ×. To the left the surface fibres of the dorsal funiculi separated by the median septum (m.s.). Blood vessels and terminal branches of a vaso-motor branch. Note the expansions of the thin nerve fibres. Bodian method.

means that they are present on both sides of the cell bodies and that, consequently, they are not influensed by a possible polarity of the cells.

The mode of termination of these vascular branches indicates a vaso-motor nature. This assumption implies that both of the cell processes of the smooth dorsal cells are axons and no additional cell processes have been observed. In

the present preparations there are no distinct indications of morphologically discernible dendrites. However, the possibility may exist that these vascular branches represent some kind of a nourishing system, establishing contact between the extra-medullar blood vessels and the smooth dorsal cells of the spinal cord. If this is the case one of the two main fibres may be a dendrite. A similar view has been expressed by TRETJAKOFF (1909) regarding the dendritic system of motor neurons.

These vascular branches have been observed only on the dorsal side of the spinal cord. In a few cases fibre branches have been followed to a position close to the lateral margin of the cord. It is possible that some of these branches proceed round the lateral margin of the spinal cord to the vessels on the ventral surface of the cord. In any case it is indicated that the vessels situated on the ventral side of the cord have a much sparser innervation than those on the dorsal side. This is difficult to interpret in combination with the fact that the vessels on the dorsal side of the spinal cord in lampreys are larger, more venous, and sparser than on the ventral side (STERZI 1904).

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b) THE CELL PROCESSES OF THE ROUGH TYPE OF DORSAL CELL

It has been observed frequently in the present material that fibres from the rough type of dorsal cells leave the spinal cord through dorsal roots. This is in accordance with the observations of previous authors (STUDNIČKA, BECCARI) although they did not distinguish between the smooth and the rough types. The fibres distributed towards the periphery from these rough cells are on an average of the same thickness as most of the fibres forming the dorsal roots. This means that some of them are very thin, other are thicker and there are all intergradations between. It has, therefore, so far proved impossible to trace these fibres through the complicated fibre bundles of the dorsal roots. For this reason the peripheral distribution of these fibres is still unknown and the nature of these fibres cannot be deduced in this way. The other of the two main fibres has been traced for long distances within the dorso-lateral funiculus. The extreme ends have not been observed. The thickness of these intra-medullar fibres of the rough dorsal cells may be the same, on an average, at that of the smooth dorsal cell processes. No branches of the vascular type have been seen on the rough dorsal cell processes. In some cases the peripheral fibre of a rough dorsal cell has been extremely thin. This indicates the possibility that the very thin visceral fibres which often accompany the thick visceral fibres (see JOHNELS 1956) may derive from such rough dorsal cells.

In addition to the main cell processes the rough type of dorsal cell has many small processes of the cell surface. These are short and often appear indistinctly impregnated (Fig. 5 A and B). They are distributed in the vicinity of the cell body in varying directions. Some of these processes can be traced a short

distance dorsally into the fibrous tissue of the adjacent funiculi. Others are directed towards the gray matter ventrally, or towards the ependyma of the central canal.

5. DISCUSSION

It has been stated previously that there are dorsal cells in lampreys which are autonomic, problay vaso-motor (JOHNELS 1956). From the description above it is evident that this is so in the case of the smooth dorsal cell type. The presence of the small branches to the extra-medullar blood vessels from both of the cell processes of this cell type makes the polarity of these cells obscure. It seems that not only is the peripheral branch efferent but also the so-called funicular branch must be efferent if the vasal branches mentioned are efferent, which seems to be the most likely possibility. However, the extreme end of the funicular fibre of the smooth cells and its connections are unknown. On the other hand it is evident that impulses must be directed to a nervous cell and the question is, then, where the dendrite system is represented in these smooth cells. In many preparations the tissue around the cell bodies of these cells reveals a multitude of very thin branches which may possibly be terminations of other neurons. These may form contacts with the surface of the smooth dorsal cells. In these preparations, however, there is a tendency towards an impregnation of neuroglia elements which makes the picture difficult to interpret. However, this contact possibility implies that no morphologically discernible dendrites are necessary on the smooth dorsal cells. It may be mentioned in this connection that there are no morphological specializations on the places of contact between the oblique fibres and the Müllerian fibres of the motor system. There is, also, the possibility that the dendrite system of the smooth dorsal cells has not been impregnated with the methods employed.

It has not been possible to trace the cell processes of the rough dorsal cells to the periphery and no information on the connections of the intra-medullar fibres is available. On the other hand the presence of the small cell processes on the cell bodies may suggest a clue as to their nature. It is very tempting to regard the small cell processes of these cells as a system of dendrites. In this case the rough cells cannot be sensory. There is a possibility that they are autonomic but the origin of the thin visceral fibres (see above) of the trunk has not so far been established.

However, it seems safe to state that there are two different types of dorsal cells in lampreys. The smooth type is characterized by a regular shape and a smooth outline of the cell body, which has two large cell processes and no or at least no obvious small cell processes representing dendrites. The nucleus has a regular ovoid shape. The smooth dorsal cell has one peripheral fibre running

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through a dorsal root in the shape of a thick visceral fibre (see JOHNELS 1956). The funicular fibre of a smooth dorsal cell has small branches directed to the extra-medullar blood vessels, and such branches may be present also on the peripheral fibre of the same cell.

The rough type of dorsal cell also has two main cell processes one of which is directed to the periphery through a dorsal root. The shape of the cell body is irregular owing to variations in the emergence of these large cell processes and, in addition, the presence of a large number of small cell processes of the cell surface. These small fibres probably represent a system of dendrites. There are no vascular branches of the large intra-medullar fibres from the rough dorsal cells. The peripheral distribution of these fibres is unknown. The nucleus of the rough dorsal cells is often irregular in shape and with a folded nuclear membrane. Inclusions are often present in the cytoplasm of rough cells.

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WHITING (1948, 1955) has studied the structure of the spinal cord in proammocoete stages of lampreys. He found cells situated in the dorsal part of the spinal cord which were regarded as being sensory and homologous with the Rohon-Beard cells of lower gnathostomes. WHITING regarded these cells of the juvenile lampreys as being the dorsal cells of the adult. That this is the case is also indicated in the work of STUDNIČKA (1895). It is a curious fact that so far no sensory cells have been observed among the dorsal cells in the present material. It is not, therefore, possible to connect the observations on juvenile stages made by WHITING with the structures of the larva or the adult as seen in the present material.

The rough dorsal cells are much more common than the smooth dorsal cells. This is in accordance with the observation (JOHNELS 1956) that the thick visceral branches of the dorsal roots are only very few in each root, there often being only a single one, or they may be missing completely. On the other hand there are many dorsal cells distributed in the spinal cord between two subsequent dorsal roots. In a paper on the electron microscopy of the lamprey spinal cord SCHULTZ, BERKOWIITZ & PEASE (1956) have discussed the structure of the dorsal cells on the assumption that they are all sensory and of the same kind. These authors have noted certain differences of the dorsal cells as compared with the large ventral motor cells of the spinal cord. It is impossible to judge which of the two kinds of dorsal cells has been studied by SCHULTZ & co-workers.

As mentioned in the introduction the dorsal cells have been regarded as exclusively sensory. The position of these cells within the spinal cord has been an argument i support of the hypothesis that the spinal ganglia of vertebrates have been formed, during phylogeny, by the emigration of ganglion cells from the spinal cord to a position outside the medulla (see ARIENS KAPPERS, HUBER & CROSBY 1936, see also WHITING 1948). Of the two types of dorsal cells present in the spinal cord of lampreys the smooth type is the one which is most

similar to the peripheral spinal ganglion cells. However, the smooth dorsal cells seem to be exclusively vaso-motor, in any case autonomic and in addition they are not bipolar. Consequently they cannot serve in the role mentioned. It may be that the function of the other type, the rough dorsal cell, is unknown, but this cell with its many small processes of the cell body surface is very different in structure from the peripheral spinal ganglion cell. The same is true regarding the shape of the nucleus, which seems to be very active in these cells. The function of a multipolar ganglion cell must be different from that of a bipolar spinal cell. It seems that in the adult or larval lamprey no cells of a spinal ganglion type are situated within the spinal cord.

6. ACKNOWLEDGMENT

The present author is deeply grateful for the extremely kind and important help of professor A. Palmgren, Veterinary College, Stockholm, concerning the solving of many of the difficult problems of silver impregnation

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7. SUMMARY

It has been demonstrated that thick visceral fibres can be traced from the periphery into the spinal cord of lampreys where the fibres originate in a particular type of dorsal cells, which has been named the smooth type. This smooth dorsal cell is characterized by a completely smooth surface of the cell body, which has a regular shape. The majority of the dorsal cells of lampreys belong to a different type, which has been termed the rough type. These rough cells are characterized by the presence of a large number of small cell processes on the surface of the cell body. Both types have two large cell processes, one of which will leave the spinal cord through a dorsal root. The peripheral distribution of this fibre originating in a rough cell is, however, unknown. From the large cell processes of the smooth cells branches are given of within the medulla towards the blood vessels on the dorsal side of the spinal cord. These fibres run dorsally in the median septum between the dorsal funiculi of the cord. No such branches have been seen on the fibres from the rough cells. The nucleus of the smooth type of dorsal cell is regularly ovoid; the nucleus of the rough type is often irregular in shape with a folded nucleus membrane. The dendritic system of the smooth dorsal cell is not morphologically discernible, at least not with the methods employed. The rough cells, on the other hand, are distinctly multipolar. No sensory dorsal cells have been observed in the present material and neither of the dorsal cell types reveal any close resemblances to the spinal ganglion cells.

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ON THE INFLUENCE OF THE FREQUENCY OF EJACULATION UPON SOME ACTIVITIES OF MALE ACCESSORY GLANDS IN BULLS AND RABBITS

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In contrast to other body fluids, such as blood, sperm plasm is not definite and constant, its composition being dependent upon the varying proportions of secretions from the different accessory glands. The secretory activity of these glands, in its turn, is influenced by manifold internal and external factors. This paper deals with the influence of the frequency of ejaculations upon some activities of the male accessory glands in bulls and rabbits, especially upon the production of the antiagglutinic factor, male sperm antagglutin, in the prostate.

MATERIAL AND METHODS

From October to May semen was collected from eight male albino rabbits by means of an artifical vagina. The frequency of ejaculations varied from once a day to once every seventh day. Each animal was given the same length of resting period between the ejaculations, at least on three occasions. Besides, a few ejaculates were collected after periods of abstinence of more than seven days.

In other experiments three ejaculates were successively collected during approximately thirty minutes. At this frequency of copulations the volumes of the ejaculates rapidly decreased, and the third ejaculate in a series was very often too small to be analysed. In order to obtain ejaculates large enough, three

rabbits yielding remarkably abundant volumes were chosen for these experiments. After collecting the ejaculates, their volumes were measured, the samples rapidly frozen (KIHLSTRÖM 1956) and stored in a refrigerator until analysed.

From eight different bulls (Swedish Red Breed), belonging to the Artificial Insemination Association at Eskilstuna thirty-four series of ejaculates were obtained, each series comprising three ejaculates collected from the same animal within half an hour.

The concentration of antagglutin in the samples was determined according to LINDAHL, KIHLSTRÖM, ROSS (1957), and KIHLSTRÖM (1958 a). In samplers large enough the percentage of solids in the sperm plasm was also determined as described earlier (KIHLSTRÖM 1958 b).

The emission of spermatozoa by rabbits during ejaculations after one, two, three, four, and seven days of abstinence has been estimated. By centifuging known volumes of semen in graded hematocrite tubes at 2300 g for ten minutes it was possible to obtain the volumes of sperm cells in the samples. On the basis of these data a calculation was made of the densities expressed as the volume of the cells in per cent of the ejaculated volume and as total amounts of sperm cells in the ejaculates.

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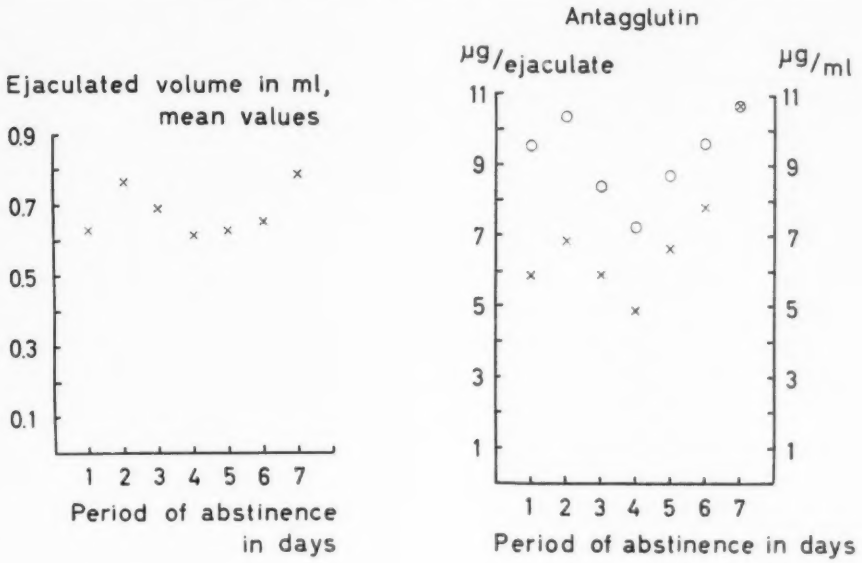
RESULTS AND DISCUSSION

In rabbits the mean volumes of the ejaculates, their mean amounts of antagglutin and total solids vary with regard to the length of the preceding period of abstinence in a manner recorded in Fig. 1 and Table I. The *mean ejaculated volume* is at a maximum when the rabbits ejaculate once every two days, and drops to a minimum when the interval between the ejaculations is prolonged to four days. The volumes of the ejaculates obtained after the interval of two days differ significantly from those yielded after the interval of four days ($t = 2.44$, $f = 133$, $p < 0.01$) as well as from those ejaculated after en abstinence of one day ($t = 2.78$, $f = 433$, $p < 0.005$). With an increase of the time of abstinence the mean volumes again rise to an interval of seven days. The volumes of the ejaculates obtained after this length of rest differ significantly from those acquired after an interval of four days ($t = 2.22$, $f = 92$, $p < 0.025$). After even longer intervals between the ejaculations the mean ejaculated volumes vary considerably (Table I), but the volumes are not statistically different.

The *amount of antagglutin* yielded per ejaculate changes with the increase of the period of abstince in the same manner as described with regard to the ejaculated volumes. Moreover, the *concentrations of antagglutin* in the semen show the same relation to the period of abstinence. (See Table I and Fig. 1 b.) After an interval of four days between the ejaculations, the concentrations of antagglutin are least, and after this interval they differ from those obtained



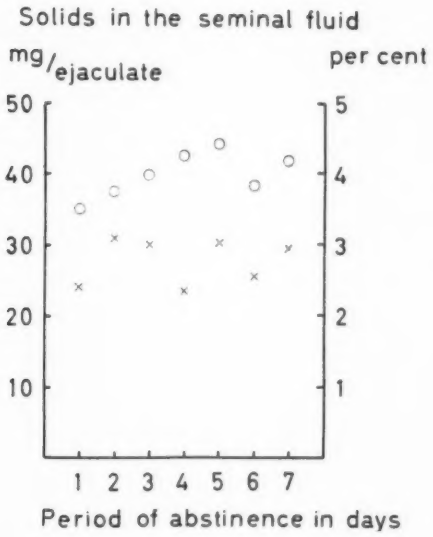


Fig. 1

a) Mean volumes of ejaculates yielded by rabbits after different periods of abstinence.

b) Mean values of the content of antagglutin in seminal fluid from ejaculates yielded by rabbits after different periods of abstinence (× = µg/ejaculate, ⊙ = µg/ml).

c) Mean values of the content of solids in seminal fluid from ejaculates yielded by rabbits after different periods of abstinence (× = mg/ejaculate, ⊙ = per cent).

TABLE I

Ejaculated volume and some properties of rabbit semen in ejaculates collected after different periods of abstinence
(n = number of ejaculates analysed)

Period of abstinence in days	Mean volume ml	n	µg of antagglutin per ejaculate	per ml	n	Solids in the seminal fluid per ejaculate	per cent	n
1	0,63±0,063	350	5,85±0,416	9,59±0,504	300	24,00±0,925	3,49±0,069	220
2	0,77±0,050	85	6,84±0,777	10,39±1,131	75	30,86±2,337	3,76±0,144	55
3	0,69±0,041	65	5,88±0,692	8,43±0,823	56	29,98±2,975	3,97±0,224	50
4	0,61±0,043	50	4,89±0,763	7,22±0,799	36	22,57±1,780	4,25±0,222	38
5	0,63±0,098	29	6,68±1,998	8,71±1,179	20	30,37±4,124	4,43±0,493	17
6	0,65±0,066	25	7,82±1,942	9,62±1,439	16	25,51±4,147	3,83±0,665	18
7	0,79±0,068	44	10,72±1,900	10,72±1,305	26	29,67±4,761	4,19±0,374	23
8	0,65±0,063	21						
9	0,55±0,114	7						
10	0,81±0,178	11						
11	0,55±0,099	5						
12	0,69±0,172	18						

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after an interval of two days ($t = 2.30$, $f = 109$, $p < 0.025$), as well as from those acquired after an interval of seven days ($t = 2.3$, $f = 60$, $p < 0.025$). There is, however, no significant difference between the values obtained from ejaculates collected after intervals of one and two days respectively.

It may be expected that the production of antagglutin in the prostate runs parallel to the production of total solids in the accessory glands, and the *mean amounts of solids* in the seminal fluid show the same dependence upon the length of the resting period as do the amounts of antagglutin (see Table I and Fig. 1 c). The maximum figures are obtained after an abstinence of two days and differ significantly from those acquired after an abstinence of one day ($t = 3.97$, $f = 273$, $p < 0.01$) as well as from those found when the interval between the ejaculations is four days ($t = 4.08$, $f = 99$, $p < 0.0005$). After even longer intervals the amount of solids in the seminal fluid again increase. The *percentage of solids* in the sperm plasm, however, continuously rises with the increase of abstinence, at least up to an interval of four days. The figures obtained after this interval differ significantly from those found after an abstinence of one day ($t = 3.30$, $f = 256$, $p < 0.001$), but not from those acquired after intervals longer than four days.

It is a well established fact that, at least in some animals (bulls, rams), matings stimulate the production of semen (see e.g. ANDERSSON 1945, pp. 45—49, ALMQUIST and HALE 1956). According to the present investigation it is obvious that in rabbits the accessory glands are most active when the interval between the ejaculations is two days. FRÖLICH (1948) has found that after this length of the resting period also the motility of the sperm cells is at maximum.

Most likely, the sexual impulses in rabbits evoked at this high frequency of ejaculations are abundant enough markedly to increase the output of ICSH from the pituitary stimulating the production of testosterone, which in its turn stimulates the activity of the accessory glands. If this be the case, however, there must be a great difference between FSH and ICSH in this respect as the output of sperm cells in the ejaculates is not influenced by frequent copulations, but continously rises when the period of abstinence increases (see Table II), which is in agreement with the figures given by FRÖLICH (1948). In bulls (LASLEY and BOGART 1943, ALMQUIST and HALE 1956) and rams (CHANG 1943), however, the spermatogenesis is shown to be stimulated by frequent copulations. After longer intervals between the ejaculations the sexual stimulation and the amount of circulating ICSH diminish, as suggested by the decrease of the volumes of the ejaculates and their content of antagglutin.

Frequently repeated ejaculations do not only stimulate the accessory glands, but also cause an exhaustion of the stored glandular products. If the period of abstinence is shorter than two days the amount of the secretion yielded in the ejaculates apparently exceeds the productive capacity of the accessory glands.

In spite of the decrease of sexual stimulation, the volumes of the ejaculates

TABLE II

The output of sperm cells in ejaculates collected from rabbits after different periods of abstinence
(n = number of ejaculates analysed)

Period of abstinence in days	Mean volume of the sperm cells per ejaculate ml	in per cent of the ejaculated volume	n
1	0,026±0,0010	5,4±0,41	10
2	0,031±0,0006	6,6±1.12	10
3	0,039±0,0045	6,8±1,15	10
4	0,047±0,0077	7,1±1,09	10
5	0,041±0,0055	7,6±0,92	10

as well as their contents of antagglutin increase after intervals of more than four days. This fact seems to indicate that also in rabbits the reproductive glands continuously produce small amounts of secretions stored in the sexual tract until an ejaculation occurs. Such a "resting secretion" was first demonstrated in the nonstimulated prostatic gland of the dog (FARELL and LYMAN 1937), and has been thoroughly studied by HUGGINS (1945). It must, however, be pointed out that, during long periods of abstinence, secretions from the accessory glands may be lost to the bladder, or in ejaculations caused by masturbation. Such losses may cause the great variability found in the volumes of the ejaculates obtained after long periods of abstinence. (See Table I.)

The well known fact that the concentrations of e.g. fructose citric acid, and acid phosphatase in the semen primarily depend upon the circulating amount of the male sex hormone shows that sexual stimulation may evoke not only quantitative, but also qualitative changes in the secretions of the male accessory glands by augmenting the production of physiologically important principles (MANN 1954). As illustrated in Fig. 1 and Table I the concentrations of solids in the sperm plasm increases with the increase of abstinence, without being noticeably affected by the sexual stimulation caused by frequent copulations. This fact may indicate that the main part of the solids are substances, the production of which is not stimulated by testosterone. If this be the case, we can look upon the percentage of solids as mainly reflecting the accumulation of materials produced during the "resting secretion" of the accessory glands.

When ejaculations occur with short intervals during approximately half an hour the capacity of the reproductive tract in storing semen is more important than the secreting activity of the accessory glands. In rabbits *the volumes of the ejaculates* diminish very rapidly as the semen stored in the genital tract is exhausted. (See Table III. Cf. also MACIRONE and WALTON, 1938.) The first ejaculates are significantly greater than the subsequent ones (first to second

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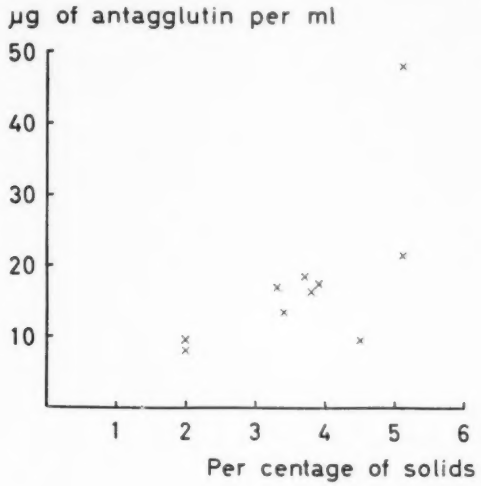

Fig. 2

µg of antagglutin per ml seminal fluid plotted against the percentage of solids. Second and third ejaculates collected from rabbits during half an hour.

ejaculate: $t = 3.45$, $f = 98$, $p < 0.0005$; first to third ejaculate: $t = 3.97$, $f = 56$, $p < 0.0005$), whereas the second and third ejaculates do not differ significantly from each other ($t = 1.27$, $f = 58$, $p < 0.2$).

The first ejaculates contain the greatest *quantities of antagglutin*. The two subsequent ejaculates show lower and approximately the same values, but owing to the small volumes of the third ejaculates their concentrations of antagglutin are greater (see Table III). The *total quantities and proportions of solids* in the sperm plasm also vary similarly.

In rabbits the seminal vesicles are absent and the secretions from the seminal glands (glandula seminalis and glandula vesicularis) and the prostatic gland together constitute the main part of the seminal fluid. As pointed out above in three successively collected ejaculates the volumes rapidly decrease, whereas the contents of antagglutin reveal only slight differences. These facts may indicate, that the loss of secretions from the seminal glands limit the number of ejaculations before the products of the prostatic gland are exhausted. In contrast to the first ejaculates of the series, the second and third show a weak positive correlation between the percentage of solids and the concentrations of antagglutin. (See Fig. 2, $r = 0.6660$, $v^2 = 6.38$, $p < 0.05$.)

Bulls have noticably large seminal vesicles containing up to 50 ml of secretory fluid (Mann 1954, p. 34), and many investigations have proved that, in series of ejaculates collected successively, the second or third ejaculate is the most abundant (Andersson 1945, p. 45, Mercier and Salisbury 1949, Almquist and Hale 1956). According to the present investigation, the volumes of the

TABLE III

Ejaculated volume and some properties of rabbit semen in three ejaculates collected from the same animal within half an hour
(n = number of ejaculates analysed)

Ejaculate number	Volume in ml	n	μg of antagglutin per ejaculate	per ml	n	Solids in the seminal fluid mg per ejaculate	per cent	n
I	0,52±0,050	49	9,17±1,515	17,97±2,098	30	18,21±5,651	3,69±0,520	7
II	0,32±0,030	51	6,42±1,000	16,22±1,329	25	12,70±1,970	3,57±0,410	6
III	0,27±0,050	9	6,71±2,252	21,01±5,303	7	12,35±2,213	3,88±0,687	4

TABLE IV

Ejaculated volume and some properties of bull semen in three ejaculates collected from the same animal within half an hour
(n = number of ejaculates analysed)

Ejaculate number	Volume in ml	n	μg of antagglutin per ejaculate	per ml	n	Solids in the seminal fluid mg per ejaculate	per cent	n
I	3,75±0,184	34	19,64±2,225	5,16±0,506	34	399,8±21,86	10,46±0,598	20
II	4,05±0,222	34	23,39±2,530	5,63±0,505	34	452,9±36,18	10,57±0,387	28
III	4,41±0,220	34	21,23±2,366	5,12±0,543	34	420,6±31,86	9,40±0,439	25

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third ejaculates are significantly greater than those of the first ($t = 2.28$, $f = 66$, $p < 0.025$). (See Table IV.)

The second ejaculate in such a series often contains most antagglutin, but there are no significant differences between the ejaculates in this respect (see Table IV). The same holds true with regard to the mean amount of solids in the ejaculates. It is interesting to compare these facts with other findings demonstrating that the second ejaculate in a series is superior to the first and third in more than one respect. Many investigators have found that the second ejaculates have the largest volumes and the greatest motility of sperm cells (ANDERSSON 1945, p. 45, MERCIER and SALISBURY 1946). Moreover DAVIS and WILLIAMS (1939) and STRÖM (1952) have demonstrated that, after inseminations, second ejaculates show a fertility slightly higher than that of the first and third ejaculates. Finally the spermatozoa of second ejaculates possess a greater mean specific gravity than those given in the first and third (LINDAHL and KIHLSTRÖM 1952).

In bull semen there is no correlation between the concentrations of antagglutin and the percentage of solids in the first three successive ejaculates.

SUMMARY

An investigation has been made into the influence of the frequency of ejaculations upon some activities of the male accessory glands in rabbits and bulls. In rabbits the volumes of the ejaculates and the production of antagglutin and total solids are at a maximum when the animals ejaculate once every two days, and at a minimum when the period of abstinence is four days, but increase at lengthier intervals.

On collecting three ejaculates successively, rabbits show a rapid decrease of the volumes ejaculated, whereas the amounts of antagglutin and total solids are nearly constant.

In series of three ejaculates successively collected from bulls the volumes are more abundant in the latter ejaculates. The concentration and amount of antagglutin as well as of total solids in sperm plasm are often greatest in the second ejaculate in such a series.

ACKNOWLEDGEMENTS

I am very much indebted to Professor P. E. Lindahl for discussions and valuable criticism. My thanks are due to Mr. A. Svensson for technical assistance and to the Swedish Natural and Agricultural Research Councils for financial support. I also thank Dr. B. Ström and the Artificial Insemination Association at Eskilstuna for semen material.

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THE VELUM AND THE RESPIRATORY CURRENT OF MYXINE

BY

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1. INTRODUCTION

As remarked by FONTAINE (1958), the course of the respiratory current in *Myxine* is unique. Water is taken in through the terminal nostril and, after passing through a very long pharynx, enters the afferent branchial ducts, passes through the gill pouches, and emerges from the efferent branchial ducts by a common aperture on each side of the body. The respiratory current is maintained, as in the ammocoete, by a pulsating velum, a fact which eluded many early authorities. Although they described the velum and its musculature, neither MÜLLER (1835) nor FÜRBRINGER (1875) realised its significance. CUNNINGHAM (1886—87 a, b), who was the first to note the course of the current, speculated at to "whether the respiratory current is maintained by ciliary action or by internal muscular action or by a combination of both". COLE (1907), in his masterly monograph, showed that the histology of the velar muscles is consistent with swift or rhythmic contraction, and concluded that "the velum may well act as an apparatus which, by a pulsating motion, draws the respiratory water into the pharynx. If this is not so, it is difficult to understand how the water reaches the gills, since the intrinsic and extrinsic musculature of the gills would only expel water from the respiratory apparatus already there".

It was left to GUSTAFSON (1935) to open a living hagfish and see the velum

in action. In a brief paper (which nevertheless contains almost all that is known of the biology of *Myxine*) he described the rhythmic motion of the velum, but without identifying the muscles or cartilages involved. MARINELLI (1956) fitted his extensive knowledge of *Myxine* anatomy to GUSTAFSON's functional description but, lacking access to living specimens, his interpretations, although mostly correct, were hypothetical. The following analysis, based on the study of living *Myxine*, is in many respects an elaboration of GUSTAFSON's account but it raises several new considerations.

2. MATERIAL AND METHODS

Hagfish were taken from 30 m. depth an the soft muddy bottom of Gullmar Fiord, off Kristineberg Zoological Station, Sweden. Some were trapped in an eel-pot baited with herring. Others were caught by GUSTAFSON's method of lowering to the bottom a 10 l. narrow-mouthed bottle filled with deep-sea water and containing bait. This method has the advantage that animals so trapped do not at any stage come into contact with surface water. It is noticeable that such animals secrete much less slime than those in the eel-pot which are exposed to water of a lower salinity as they are hauled to the surface. However, after one day in the aquaria, no difference could be detected in the activity of animals caught by either method, and none died. Two aquaria were used, one a concrete tank, $1\frac{1}{2} \times 1 \times 1$ m. and the other of glass, $60 \times 60 \times 40$ cm., the latter with 25 cm. depth of mud. Both aquaria were supplied with running water taken continuously by pipe from a depth of 30 m. and having a salinity of ca. 33 parts per thousand and a temperature of ca. 8° C.

Animals were anaesthetised when necessary by immersion in a 5% solution of ethyl urethane in sea-water for about 15 minutes. Individuals less than 15 cm. long seldom survived longer periods in the anaesthetic, but individuals 30 cm. or more in length have survived more than two hours' anaesthesia. It may be remarked that, except for its tendency to bleed, *Myxine* is a very hardy and convenient experimental animal.

Respiratory currents were detected by dropping a solution of methylene blue in sea-water into the aquarium near the animals. For some work it was found more convenient to observe the animals in about 4 cm depth of water on a white painted aquarium table.

3. RESPIRATION IN BURROWS

It is reasonable to assume that *Myxine* normally burrows in the mud on which it is found. In an aquarium provided with mud, most of the animals

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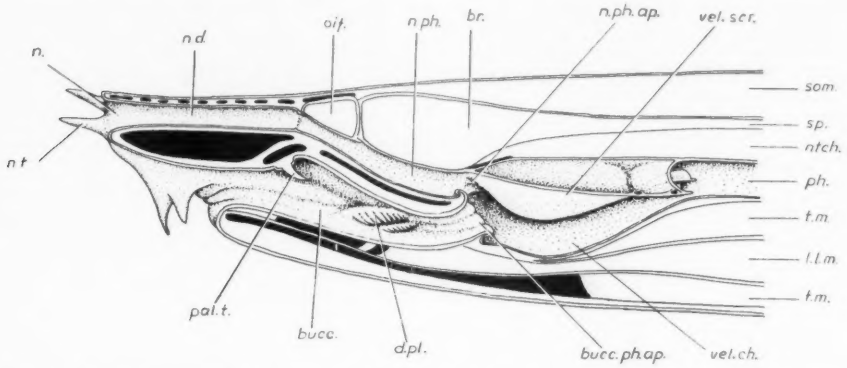

Fig. 1. Semi-diagrammatic sagittal section through the head of *Myxine*. *br.*, brain; *bucc.*, buccal cavity; *bucc.ph.ap.*, buccopharyngeal aperture; *d.pl.* dental plates; *l.l.m.*, longitudinal lingual muscle; *n.*, nostril; *n.d.*, nasal duct; *n.ph.*, nasopharyngeal duct; *n.ph.ap.*, nasopharyngeal aperture; *n.t.*, nasal tentacle; *ntch.*, notochord; *olf.*, olfactory organ; *pal.t.*, palatine tooth; *ph.*, pharynx; *som.*, somites; *sp.*, spinal cord; *t.m.*, tubular muscle; *vel.ch.*, velar chamber; *vel.scr.*, velar scroll.

formed temporary, more or less U-shaped burrows in which they lay motionless during the daytime. Usually only the four nasal tentacles (Fig. 1, *n.t.*) projected above the surface of the mud, resembling a small pink hydroid. Occasionally the mouth was exposed. The posterior aperture of the burrow lay on a small conical eminence and communicated by a vertical tube or fissure with the burrow.

The inhalant current is very weak and is imperceptible more than 5 mm. from the edge of the nostril. Under good viewing conditions it is seen to be periodic, having a frequency of 11—15 pulses per minute.

If coarse particles of mud enter the nostril, the animal responds by a violent 'sneeze' which projects particles 3—4 cm. from the nostril. If the irritation is prolonged, the animal withdraws its head below the surface of the mud and does not reappear for several minutes. One animal withdrew into the mud for 12 minutes.

The exhalent current is continuous, but occasionally augmented by a vigorous spurt which expels large suspended particles from the burrow. In response to reduced oxygen content of the water, the animals emerge further from their burrows and eventually come to lie on the surface of the mud. As noted by GUSTAFSON, hagfish are markedly nocturnal and leave their burrows at night.

4. STRUCTURE AND MECHANICS OF THE VELUM

The velum is suspended from the dorsal wall of the pharynx by a longitudinal double membrane, the velar frenulum, from which arises a horizontal membrane

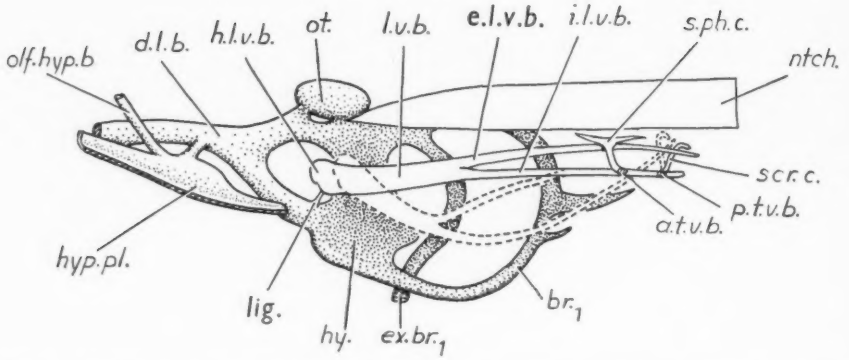

Fig. 2. Skeleton of *Myxine* in the region of the velar chamber, left side removed. Dotted lines show the extent of movement of the lateral velar bars. *a.t.v.b.* anterior transverse velar bar; *br.*$_1$, first branchial arch; *d.l.b.*, dorsal longitudinal bar (cut); *e.l.v.b.*, external lateral velar bar; *ex.br.*$_1$, first extrabranchial arch (cut); *h.l.v.b.*, head of lateral velar bar; *hy.*, hyoid arch; *hyp.pl.*, hypophysial plate (cut); *i.l.v.b.*, internal lateral velar bar; *lig.*, ligament of head of lateral velar bar; *l.v.b.*, lateral velar bar; *ntch.*, notochord; *olf.hyp.b.*, bar connecting olfactory capsule to hypophysial plate (cut); *ot.*, otic capsule; *p.t.v.b.* posterior transverse velar bar; *scr.c.*, scroll cartilage; *s.ph.c.*, suprapharyngeal cartilage.

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(like the cross-bar of an inverted "T"), the free edges of which curl dorsally in the resting organ. The term, *velar scrolls* (Figs. 1, 4, *vel.scr.*), is here applied to these lateral portions. Each scroll is supported by a lateral velar bar (Fig. 2, *l.v.b.*), the posterior two-thirds of which bifurcates to form the external lateral velar bar (Figs. 2, 3, *e.l.v.b.*), supporting the free lateral edge of the scroll, and the internal lateral velar bar (*i.l.v.b.*) which supports the internal margin of the scroll. Each internal lateral bar is attached to its partner on the opposite side by an anterior transverse velar bar (*a.t.v.b.*) and a posterior transverse velar bar (*p.t.v.b.*). From the middle of the anterior transverse bar a paired member, the suprapharyngeal cartilage (*s.ph.c.*), passes dorsally within the frenulum. Its branching halves lie on the dorsal wall of the pharynx, embedded in the connective tissue close to the notochord, on either side of the dorsal aorta. The suprapharyngeal cartilage is the only fixed element in the velar skeleton. However, its attachment to the square area enclosed between the anterior and posterior transverse bars and the internal lateral bars, renders this relatively rigid also.

The anterior end of each lateral bar is expanded into a head (Fig. 2, *h.l.v.b.*) which is attached by a ligament (*lig.*) to the anterior border of the hyoid arch. The lateral bar is thus free, not only to rotate, but also to move within the radius of the ligament. However, the relatively fixed position of the internal lateral bar imposes a restraint, and it can be seen from figs. 2 and 3 that only the external lateral bar has real freedom of movement.

Four muscles control the movement of the lateral bar. The posterior cranio--velar muscle (Fig. 3, *p.c.v.m.*) originates on the hypophysial plate (*hyp.pl.*)

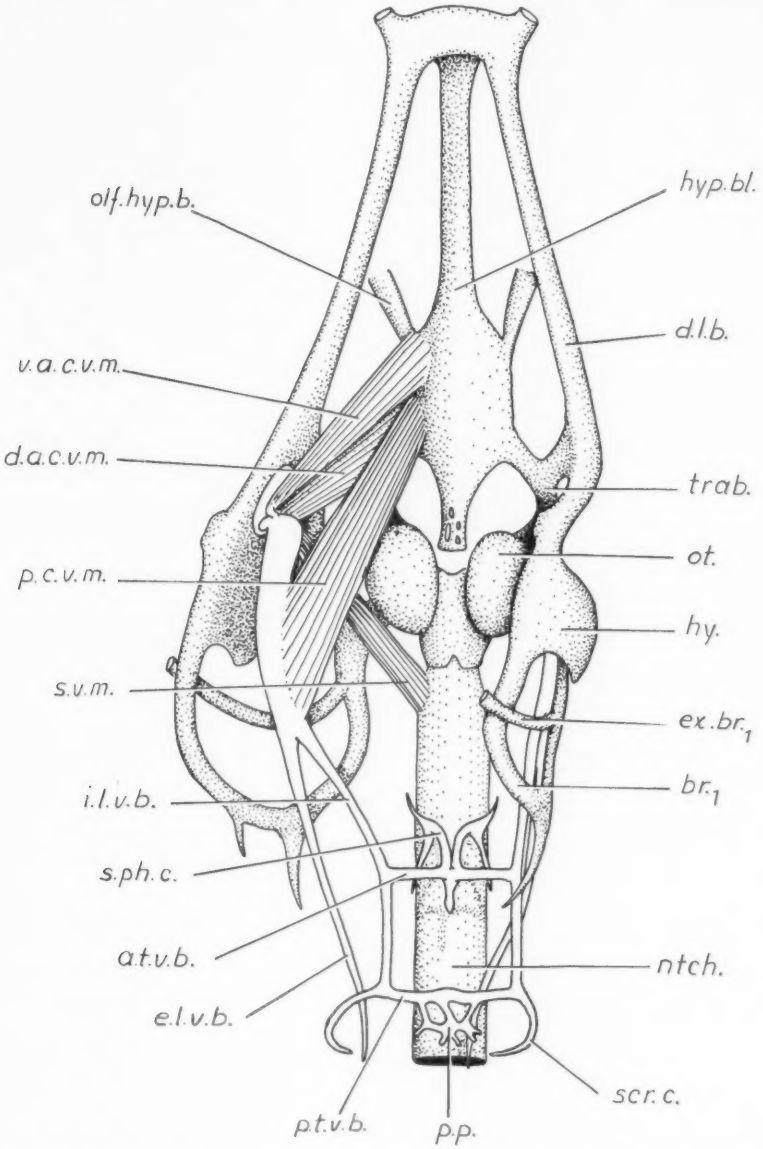

Fig. 3. Ventral view of the skeleton in the region of the velar chamber. The right side has been pulled outwards to display the velar muscles. *d.a.c.v.m.*, dorsal anterior cranio--velar muscle; *p.c.v.m.*, posterior cranio-velar muscle; *p.p.*, posterior process of posterior transverse velar bar; *s. v. m.*, spino-velar muscle; *trab.*, trabecula; *v.a.c.v.m.*, ventral anterior cranio-velar muscle. Other abbreviations as in Fig. 2.

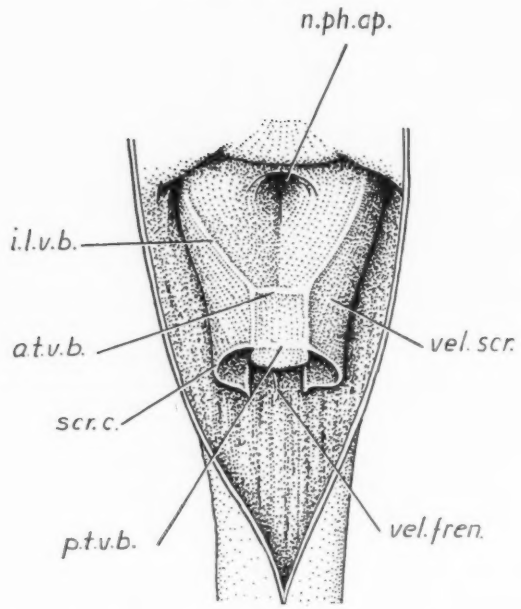

Fig. 4. Ventral view of velum, ventral wall of velar chamber opened. *a.t.v.b.*, anterior transverse velar bar; *i.l.v.b.*, internal lateral velar bar; *n.ph.ap.*, nasopharyngeal aperture; *p.t.v.b.*, posterior transverse velar bar; *scr.c.*, scroll cartilage; *vel.fren.*, velar frenulum; *vel.scr.*, velar scroll.

and is inserted on the ventro-mesial face of the lateral bar, just anterior to its bifurcation. Contraction of this muscle tends to pull the lateral bar antero--mesially. However, the restraint imposed by the internal lateral bar will only permit rotation of the system so that the external lateral bar comes to lie internally and ventrally to the internal lateral bar. When the posterior cranio-velar muscles of each side are fully contracted, each external lateral bar meets its partner tangentially below the velar frenulum.

Three muscles work antagonistically to the posterior cranio-ventral muscle. The dorsal and ventral anterior cranio-velar muscles (*d.a.c.v.m.*, *v.a.c.v.m.*) originate on the hypophysial plate and are inserted on the head of the lateral bar. Their contraction returns the external lateral bar to its resting position. The spino-velar muscle (*s.v.m.*) has a similar but more marked effect. By pulling the head of the lateral bar medio-caudally, it accentuates the curvature of the lateral bar. Thus, when rotated dorsally under the combined action of the anterior cranio-velar and spino-velar muscles, the lateral bar comes much closer to the wall of the pharynx than when it rotates ventrally under the pull of the posterior cranio-velar muscle.

On each side of the posterior transverse bar arise two curved cartilages, here

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termed the *scroll cartilages* (*scr.c.*), which reach almost to the posterior tips of the external lateral bars. The velar scrolls, supported between the external and internal lateral bars, and posteriorly by the scroll cartilages, have their curvature imposed upon them by the latter elements.

The anterior part of the pharynx is expanded into a cavity, here termed the *velar chamber* (Fig. 1, *vel.ch.*), supported on all sides by the visceral skeleton. As indicated in figs. 2 and 5, the hyoid (Fig. 2, *hy*), first branchial arch ($br._1$), and first extrabranchial arch ($ex.br._1$) contribute to the framework. The posterior processes of the first branchial arch are also involved in forming a rigid basket surrounding the velum. It will be seen that this rigidity is esssential to the functioning of the velum.

The nasopharyngeal duct (Fig. 1, *n.ph.*) opens into the pharynx at the origin of the velum, and the buccopharyngeal aperture (*bucc.ph.ap.*) lies just below it. However, whereas the nasopharyngeal duct is a permanently open tube, the buccal cavity is closed when the dental apparatus is inverted.

5. FUNCTION OF THE VELUM

Fig. 5 shows four successive stages in the velar cycle. This was elucidated partly by observation of the velum through the translucent walls of the velar chamber after removal of the somatic musculature of one side, and partly by opening the ventral wall of the velar chamber. The contraction of the velar muscles was observed after removal of parts of the epithelial membrane of the velum.

Fig. 5 A shows the resting position of the velum. This position is taken up in deep anaesthesia or in death, and is presumably maintained by the elasticity of the cartilages. In fig. 5 B, the velar scrolls are beginning to unroll ventrally under the pull of the posterior cranio-velar muscles. In fig. 5 C they have reached their maximum extension, the inwardly bowed lateral bars of each side meeting in the mid-line. This movement is relatively slow and, since the scrolls do not extend as far as the walls of the velar chamber, the water contained in it is only slightly disturbed. On the return excursion (shown in Fig. 5 D) the movement is much faster* and the external bar is extended outwards by the action of the spino-velar muscle and touches the ventro-lateral walls of the velar chamber. As a result of this, most of the water in the velar chamber is transported upwards within the scrolls and deflected posteriorly by the velar frenulum. At the same time, water flows in through the nasopharyngeal aperture, occupying the space below the velum. It seems likely that the scroll cartilages,

* It may be noted that this was predicted by MARINELLI (1956): "Es ist sehr wahrscheinlich, dass das Ausbreiten der Velarflügel langsam, das Einrollen rasch geschieht, aber darüber stehen Beobachtungen noch aus".

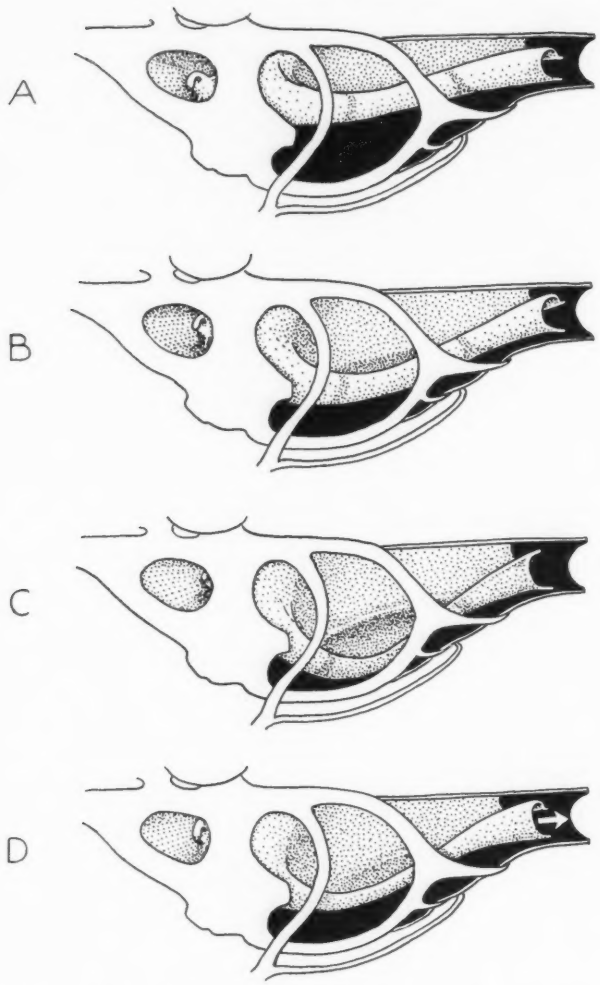

Fig. 5. Four successive stages in the velar cycle. The velar chamber is shown from the left side with the left pharyngeal wall removed. A, resting; B, velar scroll beginning to unroll; C, velar scroll unrolled to full extent; D, velar scroll beginning to move dorsally, arrow showing direction of water flow.

holding the posterior border of the velum against the walls of the pharynx, prevent the simple return of the water into the velar chamber. The posterior process (Fig. 3, *p.p.*) may also assist this function.

The major point in which this account differs from that of MARINELLI (1956) is in the function of the spino-velar muscle. MARINELLI suggested that it assisted the posterior cranio-velar muscle in unrolling the velar scrolls.

GUSTAFSON (1935) has shown that neither the gill pouches nor their ducts

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play an active part in maintaining the respiratory current. It may be added that the pharynx, posterior to the velar chamber, is normally a flaccid tube which is passively expanded by each injection of water from the velar chamber. This, together with the resistance offered by the gills themselves, damps the rhythm of the respiratory current so that water issues from the external branchial apertures in a continuous stream. This observation, based on the behaviour of anaesthetised and dissected animals, is also true of the intact *Myxine*.

The frequency of velar movements varies considerably between individuals and at different times in the one animal. GUSTAFSON (1935) recorded 50—100 pulses per minute in active individuals, and not more than 25—30 in "sleeping" animals. The frequency range observed in the present experiments was 38—82 pulses per minute. As mentioned above, animals in burrows appeared to have a frequency of 11—15 pulses per minute, but it is possible that this is an under--estimate.

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6. "SNEEZING"

It has been mentioned above that *Myxine* responds to inhalation of large particles by a violent expulsion of water through the nostril, here termed a "sneeze". The mechanism of sneezing remains obscure. A preliminary hypothesis that it was due to contraction of the nasal duct itself was disproved by removing the dorsal wall of the duct as far back as the olfactory capsule; this operation did not impair the sneezing response. The hypophysial plate is seen to move rhythmically with the velum, but this appears to be incidental to the contractions of the cranio-velar muscles. No muscles exist which could move the hypophysial plate dorsally so as to compress the naso-pharyngeal duct. The structure and known function of the velum makes it highly unlikely that it could reverse its activity so as to pump water anteriorly. Possibly the mechanism resides in the pharyngeal constrictor muscle which encircles the posterior part of the velar chamber. Sudden contraction of this muscle would certainly reduce the volume of the velar chamber and if, by pressing the pharyngeal wall against the scroll cartilages, the posterior pharynx were sealed off, the effect would be to drive water outwards through the nasopharyngeal duct.

7. FUNCTION OF THE PHARYNGEO-CUTANEOUS DUCT

Many misconceptions of the morphology of myxinoids have been accepted uncritically by successive authors since MÜLLER's (1834) pioneer monograph. One such error is embalmed in the term, "oesophageo-cutaneous duct" given to the tube leading from the enteron to the exterior on the left side of the body.

This structure is homologous with the gills and has the same embryological origin (HOLMGREN, 1946). Unless it be assumed that gill pouches arise from the oesophagus (and the very existence of an oesophagus in *Myxine* is open to question), the organ must be a *pharyngeo-cutaneous duct,* which term is here introduced.

The pharyngeo-cutaneous duct, being wide and short, offers less resistance to the passage of water than the gill pouches and their ducts. Therefore it must be closed to allow the normal flow of water through the gills. When a resting animal is lying on its side, a steady stream of water is seen to be projected postero-laterally from the common aperture of the pharyngeo-cutaneous duct and the left branchial aperture. This stream comes from the gills. Occasionally there is a sudden contraction of the branchial region and a jet of water issues from the common aperture, almost at right-angles to the body. This stream is from the pharyngeo-cutaneous duct, and is apparently due to contraction of the branchial and cardiac constrictor muscle and simultaneous relaxation of the sphincter of the pharyngeo-cutaneous duct. The action of the branchial and cardiac constrictor muscle is to compress both the gills and the pharynx so that although most of the contents are ejected through the pharyngeo-cutaneous duct, there is also an increased spurt through both branchial apertures. It is noticeable that the particles expelled through the pharyngeo-cutaneous duct are larger than those emerging from the gills, and it seems that the function of the duct is simply to permit periodic flushing of the pharynx, clearing it of particles too large to enter the afferent branchial ducts. This function has also been suggested by MARINELLI (1956).

It has been suggested (see Section 9) that in exceptional circumstances the pharyngeo-cutaneous duct might permit the entry of water into the pharynx. To test this possibility, the nasal ducts of three animals were occluded by fitted wooden plugs held in position by fine sutures. After 24 hours, when the animals appeared to have recovered from the operation, each was observed for a period of 2 hours. Drops of methylene blue were introduced into the water near the external branchial apertures but no currents, either ingoing or outgoing, were detected. Nor could any current be seen to enter the mouth, although pulsations of the body wall indicated that the velum was in operation. Observation for shorter periods on subsequent occasions revealed no sign of a respiratory current. The plugs became dislodged from two of the experimental animals after two days, but the third individual survived with its nostril occluded for 12 days, during which time it swam vigorously when disturbed, but appeared to rest for longer periods than did intact animals. On the 13th day, the plug became dislodged. When again observed, the animal was found to be passing the normal respiratory current.

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8. CUTANEOUS RESPIRATION

The demonstration that *Myxine* can survive with its nostril occluded is not, at first sight, surprising. On anatomical grounds it would seem possible for the respiratory current to enter through the mouth. If the dental plates are everted, a wide channel is open to the velar chamber, but the experimental animals were never observed to take advantage of this opportunity. Thus it seems that *Myxine* can obtain sufficient oxygen—at least for basal metabolism—through the skin. When one considers the extreme vascularity of the skin, the rapid circulation effected in the caudal fin by the caudal heart, together with the fact that hagfish are inactive for long periods, this is perhaps understandable.

GUSTAFSON (1935) ascribed the death of hagfish exposed to surface water (60 % of the salinity of deep-sea water) to asphyxia. Animals exposed to this low salinity secreted copious slime, their nasal ducts became blocked, and they subsequently died. Removal of the slime from the nasal duct promptly restored the respiratory current. It now appears that three factors contribute to the death of animals in surface water: (1) osmotic stress *per se;* (2) obstruction of the respiratory current, and (3) diminution of cutaneous respiration by the slime surrounding the animal. It seems that slime secretion is an indiscriminate response to irritation and that in this situation it is non-adaptive.

9. RESPIRATION WHILE FEEDING

When *Myxine* has its head embedded in a fish it cannot pass a stream of oxygenated water through the nasal duct to the pharynx and gills. PUTNAM (1873—74), JORDAN & SNYDER (1901), SEDGWICK (1905) and RAUTHER (1937) have suggested that this difficulty is overcome by intake of water through the pharyngeo-cutaneous duct. NORMAN (1931) sees no such difficulty: "*Myxine* . . . bores right into the fishes it attacks. Under these conditions, the current of water is inhaled through the single nostril situated on the top of the head". SEDGWICK (1905), on the other hand, observed that "the nasal passage is a narrow one and perhaps hardly sufficient to supply all the respiratory water . . . it would appear more probable that some, at least, of the inspiratory water enters through the oesophageo-cutaneous duct". GOODRICH (1930) does not involve the pharyngeo-cutaneous duct in his explanation: "Breathing takes place by the expansion and contraction of the muscular walls of the (branchial) sacs. When the Myxinoid is feeding and its head is buried in the prey, water may still pass in and out by these tubes. It is no doubt to facilitate this respiration that the gill sacs in Myxinoids are situated very far back and the expiratory tubes lengthened so as to carry the external apertures still further from the head". A similar point of view is expressed by LAMEERE (1932—43): "(les

Myxinoïdes) ne se contentent pas de sucer ceux-ci (autres Poissons), comme le font les Pétromyzontoïdes, mais ils y enfoncent l'extrémité anterieure du corps, ce qu'ils peuvent faire grâce au recul de leur région respiratoire".

All of the above-named authors, except RAUTHER, neglect the function of the velum and assume, tacitly or explicitly, that the gill pouches are capable of independent pumping movements. In the light of present knowledge, these explanations are untenable. Of modern authors who have considered the problem at all, only MARINELLI (1956) has perceived the difficulties of the proffered solutions: "Es ist noch nicht klar, wie bei diesem sehr geringen Wasserdruck eine Zirkulation aufrechterhalten werden kann, wenn das Tier bei der Nahrungsaufnahme mittels seines Schlingapparates den probranchialen Darm gefüllt hat. Eine Stockwerksgliederung, wie sie uns im Darm von *Lampetra* durch die Ausbildung des Wasserganges bekannt wurde, oder eine Einrichtung durch einen rückläufigen Wasserstrom durch die äusseren Kiemenporen nach innen die Atmung aufrechtzuerhalten, wenn der Weg von der Nase her verstopft ist, wie sie ebenfalls bei *Lampetra* besteht, ist hier nicht nachzuweisen oder auch nur wahrscheinlich zu machen". Mention of food in the prebranchial gut is very relevant. This would interfere not only with the function of the velum, but also with any possible flow through the pharyngeo-cutaneous duct. The relaxation of the cardiac constrictor muscle, allowing flow into the intestine during feeding, would also be a complicating factor.

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Since cutaneous respiration supplies at least enough oxygen for minor activity, *Myxine* need not lack oxygen when it begins to feed. However, an oxygen shortage may supervene in the course of feeding, which is the most energetic activity undertaken by the animal. Not only is the massive dental and lingual musculature brought into operation, but the animal must also swim vigorously to maintain contact with the tissue which it is biting. It may be for this reason that the period spent with the head embedded in a fish is so short. I have no quantitative data on this but my impression, when attempting to photograph feeding animals, was that no individual fed for more than 3 or 4 minutes. It would be of great interest to know the extent to which *Myxine* can support an oxygen debt.

10. PHYLOGENETIC CONSIDERATIONS

GOODRICH (1930) is of the opinion that the posterior position of the gills and their ducts in *Myxine* is an adaptation permitting respiration while the head is embedded. In the absence of this function, another explanation must be sought. The branchial region begins where the large tongue muscles end, and it seems reasonable to assume that the gills have been displaced by the muscles. The adaptive value of a posterior migration of the efferent branchial apertures

is perhaps to be found in the burrowing habit of Myxinoids. It is advantageous for a burrowing aquatic animal to have the exhalant current directed away from the inhalant current so as to minimise re-circulation. However, the present disposition of the branchial ducts may be an ancient character. According to STENSIÖ (1958), the myxinoids are descended from Heterostraci, a group which already possessed common branchial apertures on each side, and also paired pharyngeo-cutaneous ducts, derived from reduced posterior gill pouches.

Myxinoids and larval lampreys are the only living craniates in which the respiratory current is maintained by a velum. The organs appear very different in the adults, but each lies at the junction of the buccal cavity and pharynx, is innervated by the trigeminus, and has a somewhat comparable musculature and skeletal attachement (LINDSTRÖM, 1949; JOHNELS, 1948). While these facts might be adduced as evidence of a common ancestral pattern, it must be realised that a functional complex of this nature could not have existed in *all* the Ostracoderms.

STENSIÖ (1958) can find no evidence of a velum in the Orthobranchiata (e.g. *Mimetaspis*) or the Oligobranchiata (e.g. *Trematapis*), in which groups the premandibular gill pouches are well developed. In the Nectaspiformes (e.g. *Nectaspis*), a strong transverse dorsal ridge lies immediately posterior to the reduced premandibular gill pouches, and is assumed to have served for attachment of the velum. The Anaspida (e.g. *Pterolepis*) lacked premandibular, mandibular, and hyoid pouches and, according to STENSIÖ, possessed a velum derived from the mandibular region. The Heterostraci (e.g. *Pteraspis*) lacked only the premandibular pouch, so the velum (which STENSIÖ assumes to have been present) must have been comparable with that of the Nectaspiformes. Thus, if the myxinoids arose from the Heterostraci, it follows that a "mandibular" velum must have arisen independently in the myxinoids and petromyzonts, and possibly also in the Anaspida. The progressive backward extension of the stomodeum in the phylogeny of these groups was accompanied by the loss of the most anterior gill pouches, the nervous, skeletal and muscular elements of which thereby became released from their respiratory functions and available for incorporation into other organs. Thus, the incorporation of mandibular elements into the velum of myxinoids and petromyzonts could be an example of convergent evolution.

11. SUMMARY

Myxine burrows in mud with only the nostril projecting above the surface. The respiratory current is maintained entirely by the velum, the structure and movements of which are described. Water enters only through the nostril. Large mud particles entering the nasal duct are violently expelled. If the nostril is

experimentally blocked, no water enters the gills, either from the mouth or from the posterior apertures. The pharyngeo-cutaneous duct serves only for the expulsion of accumulated particles from the pharynx. *Myxine* can apparently obtain enough oxygen through its skin to sustain minor activity. It appears that Myxine, when feeding, has no respiratory current. It is suggested that the posterior position of the branchial region is due to displacement by the tongue musculature and that the backward direction of the gill ducts is an adaptation to the burrowing habit. It is likely that the velum has originated independently in the petromyzonts and myxinoids.

I wish to express my thanks to Dr. G. Gustafson of the Kristineberg Zoological Station for providing the many facilities which made this work possible and to Prof. E. Stensiö for his valuable advice on palaeontological questions.

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THE EARLY ONTOGENY OF THE SENSORY LINES AND THE DERMAL SKULL IN *POLYPTERUS*

BY

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INTRODUCTION

In an earlier paper the author (PEHRSON, 1947) advanced some partly new interpretations of the morphology of the dermal skull in *Polypterus*. The results were founded on an examination of a series of comparatively young stages, collected by the first Swedish Gambia expedition in 1932—33. The youngest specimen then available measured 24 mm in total length. With the exception of the very young stages collected by BUDGETT, briefly described by KERR (1907) and later re-examined as far as the chondrocranium is concerned by MOY-THOMAS (1933), the 24 mm specimen was, at that time, the youngest described. The material at hand did not, however, allow a study of the earliest primordia of the dermal bones in the skull, and several problems had to be left to future work.

An opportunity offered itself when the second Swedish Gambia expedition succeeded in capturing one specimen of *Polypterus senegalus* (?) (JOHNELS, 1954), measuring only 9.6 mm. As not even this specimen seemed to fulfil the

expectations of settling all problems, the three young BUDGETT specimens were placed at the author's disposition by the courtesy of Professor C. M. YONGE, University of Glasgow. They were collected in 1900 and sectioned by the late Professor GRAHAM KERR. As is known, the two bigger specimens measure 9.3 and 8.0 mm in total length respectively, and the third and smallest specimen 6.75 mm. This specimen is sectioned sagittally, the two others being cross--sectioned. They are still in a fairly good condition, although slightly faded round the edges of the cover-slips. Unfortunately the first slide of the 8 mm specimen has not been available to the author. As mentioned by MOY-THOMAS (l.c.) the left-hand side of this specimen was damaged before sectioning.

The chondrocranium of the 9.6 mm specimen differs so little from the conditions in the 9.3 mm specimen already described by MOY-THOMAS that no special attention has been paid to it in this paper.

In the 24 mm specimen described by the author there are only two ossifications in the neural cranium, the sphenoid and the occipital. The 9.6 mm specimen shows none. Incipient perichondral ossification shows only in the splanchnocranium.

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THE SENSORY LINE SYSTEM

The development of the sensory line system from the 24 mm stage and upward has already been described by the author (1947). Typical of *Polypterus* is, as earlier described (PEHRSON 1947), the constant number of sense organs in the sensory canals, whereas the number of neuromasts in the pit line system varies somewhat (JARVIK, 1947).

CANAL ORGANS

In none of the four larval stages described in this paper is there any indication of the forming of the future sensory canals. The sense organs are still lying quite superficially, and no difference in size or structure between the future canal organs and the pit line organs can be discerned.

Already in the 6.75 mm specimen (BUDGETT's stage 32) most of the future canal organs are formed (Figs. 1 and 2). The *supraorbital line* consists of the six organs forming the line in the adult. The first organ differs from the rest by having a ventral position on the snout. The space between this organ and the dorsally placed one is also slightly bigger than between the others. In the adult this organ is situated in the first nasal or terminal bone, and the portion of the supraorbital canal in this bone turns laterad and slightly caudad. This circumstance has made JARVIK (1942 and 1947) suggest that the terminal canal portion is the vestige of a supposed anterior transverse commissure between the supraorbital and the infraorbital canals. For comparative anatomical reasons

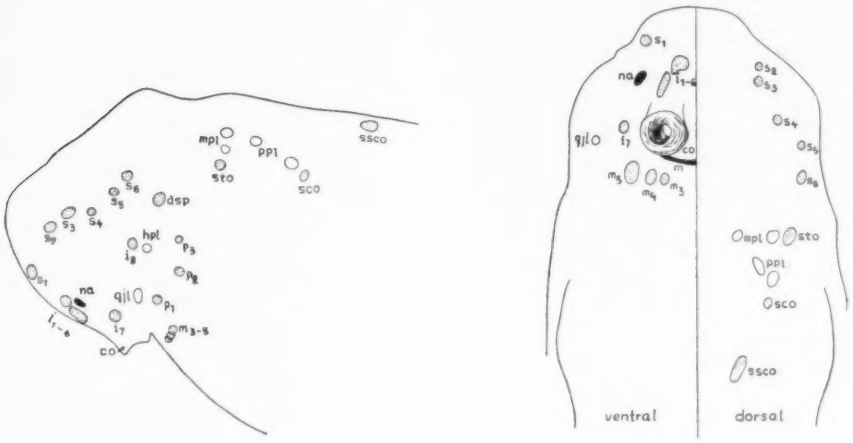


Fig. 1. *Polypterus*, 6.75 mm. Reconstruction of lateral view. For lettering see p. 258.

Fig. 2. *Polypterus*, 6.75 mm. Reconstruction of ventral and dorsal view.

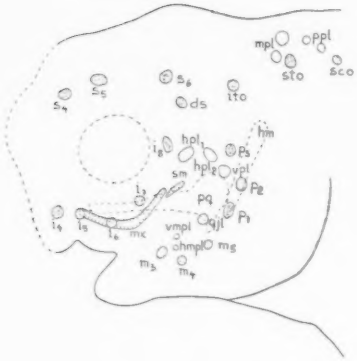
the author (1947) has expressed his doubts about this theory. Even if the terminal organ admittedly has a position by itself already at this early stage there seems to be nothing indicating any form of connection between the two canals.

As to the 8 mm specimen (BUDGETT's stage 33), the first slide with the anterior end of the snout is missing. The first organ to be observed in the second slide is, in the supraorbital series, No. 4 (Figs. 3 and 4).

In the 9.6 mm specimen as well as in the 9.3 mm (BUDGETT's stage 36) the rostral end of the supraorbital line has assumed the shape of the adult. The first supraorbital organ has, thus, a position lateral and slightly caudal to the second organ (Figs. 7 and 8).

In the *infraorbital line* in the 6.75 mm specimen the rostral and the suborbital portions are represented by two placode-like epithelial thickenings, corresponding to the first six organs (Figs. 1 and 2). The comparatively late development of this portion of the line is no doubt due to the still functioning so-called cement organs. Their basal parts partly occupy the space where the anterior and middle portions of the line will be situated. In the postorbital section the organs Nos. 7 and 8 as well as the future dermosphenotic organ are seen.

In the 8 mm specimen the infraorbital organs Nos. 4 and 5 have developed (Fig. 3). Whether the first three organs have also formed cannot be observed, owing to the missing first slide. At later stages all infraorbital organs are developed, and the line shows nothing of particular interest. No indication of any connection or anastomosis with the supraorbital line can be observed. As to the otic part of the line, no trace of the future intertemporal organ can be found in the 6.75 mm specimen, whereas it is well developed at later stages.



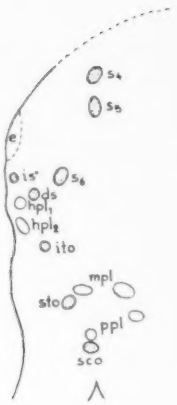


Fig. 3. *Polypterus*, 8 mm. Reconstruction of lateral view.

Fig. 4. *Polypterus*, 8 mm. Reconstruction of dorsal view.

Still further caudad in the 6.75 mm specimen the supratemporal organ, the lateral organ in the supratemporal cross commissure (extrascapular organ No. 3) and the future suprascapular organ are developed.

As to the *mandibular line*, only the canal organs Nos. 3, 4 and 5 are developed in the 6.75 mm specimen and that is also the case in the 8 mm specimen (Figs. 1, 2, 3). In the youngest specimen the mouth is only a transverse slit and the lower jaw is very undeveloped. Even in the 8 mm specimen the lower jaw is very short, which explains the late development of the anterior end of the mandibular line.

The *preopercular line* is already formed in the youngest specimen, and consists of three organs, as in the adult (Figs. 1, 2).

PIT LINES

As might be expected the pit line system is very little developed at the early stages of *Polypterus* described in this paper. It is typical that the different lines are, as a rule, represented only by one single neuromast, rarely by two sense organs.

The *nasal pit line* does not show yet in the youngest specimens. In the 9.3 specimen as well as in the 9.6 mm one it is represented by a single neuromast situated caudally to the supraorbital canal organ No. 1 (Figs. 7, 8). JARVIK (1947) suggests the possibility that this pit line—called by him the tectal line—might represent the pit line component of his anterior transverse commissural sensory line. Nothing in the ontogeny seems to indicate any special morphological importance of this line.

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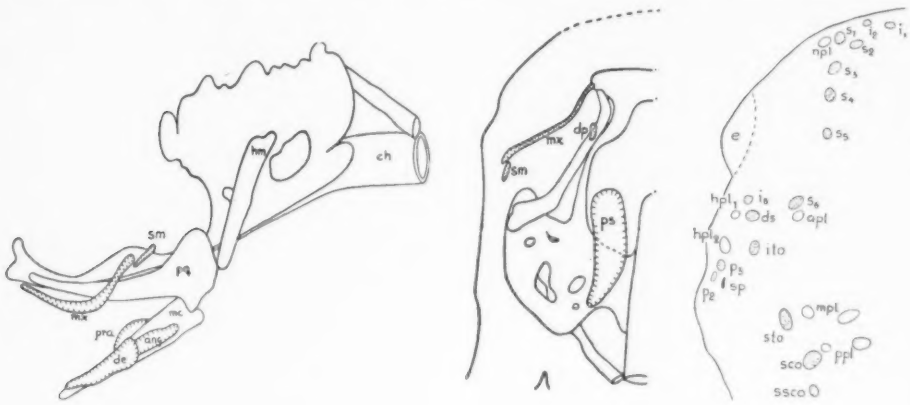

Fig. 5. *Polypterus*, 8 mm. Reconstruction of skull in lateral view.

Fig. 6. *Polypterus*, 8 mm. Reconstruction of skull in ventral view.

Fig. 7. *Polypterus*, 9.3 mm. Reconstruction of dorsal view.

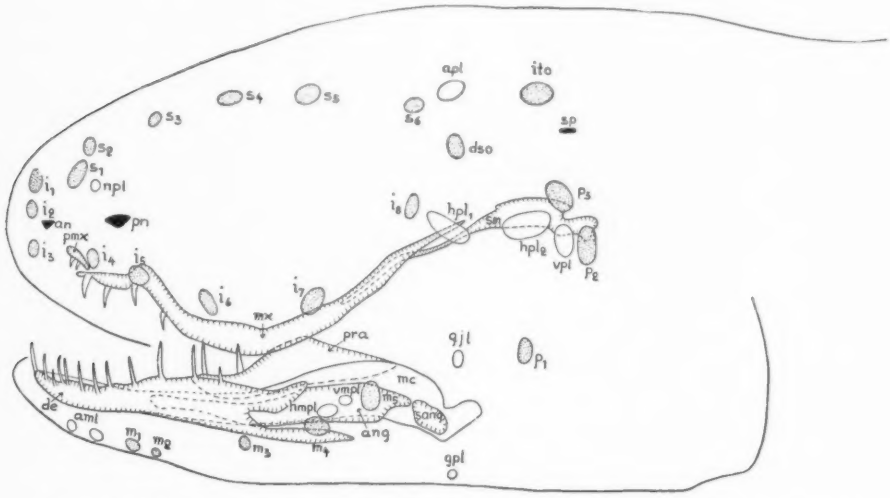

Fig. 8. *Polypterus*, 9.6 mm. Reconstruction of sensory lines and dermal skull in lateral view.

Also the *anterior pit line of the head* develops comparatively late. Nothing is to be seen of it either in the 6.75 mm specimen or at the 8 mm stage. In the 9.3 mm specimen as well as in the 9.6 mm one the anterior pit line consists of a single sense organ, lying immediately caudally and slightly medially to the sixth supraorbital canal organ (Fig. 7). In the 24 mm specimen it is formed by two sense organs (Pehrson 1947). The late development and the insigni-

ficant size of this line, even in adult specimens (JARVIK, 1947), indicate that it is, if not exactly rudimentary, yet to be considered a line in reduction.

On the top of the head there are, in *Polypterus,* still two more lines of pits, the *middle* and the *posterior pit lines.* Both are developed already in the 6.75 mm specimen (Figs. 1, 2). Earlier (1947) the author demonstrated that while the anterior line of pits is innervated by a ramification from the same branch of the facialis nerve as the 6th supraorbital organ, the middle line is innervated by the branch of the glossopharyngeus nerve that also supplies the supratemporal canal organ. The posterior line gets its nerve from the vagus branch that runs to the supratemporal canal organ. Already these circumstances seem sufficient to establish the independence of the posterior line. HOLMGREN (1942) regarded this line as homologous with the posterior line in other fishes, although the position of the line in the adult makes it appear only as a subdivision of the middle line. JARVIK (1947) is inclined to agree with the older interpretation of the line as only a portion of the middle line. It is then of some interest to consider the ontogeny of the two lines in question.

In the 6.75 mm specimen there is, immediately medially to the future supratemporal canal organ a short transverse line consisting of two sense organs (Figs. 1, 2). This is the middle line of pits. The position as well as the number of sense organs is the same in the 8 mm specimen (Fig. 4). In the 9.3 and the 9.6 mm specimens the line still consists of two sense organs, the only difference being that the line has moved slightly rostrad (Fig. 7). Later, as in the 24 mm specimen (PEHRSON, 1947), it has altered its position still more rostrad, and has reached its definite place immediately medially to the canal pore opening between the intertemporal and the supratemporal organs.

As to the posterior line of pits, it consists in the 6.75 mm specimen also of two sense organs, forming a line running in a rostro-medial direction from a point slightly rostrally to the extrascapular organ (Figs. 1, 2). At the later stages described here the position of the line and the number of sense organs are the same. In the 24 mm specimen the posterior line of pits has moved rostrad and mediad, and runs as a medial prolongation of the middle line of pits. That is the case also in a 27 mm specimen. Not until the 37 mm stage do the two lines begin to attain the position they occupy in the adult, and, at the same time, the number of sense organs begins to multiply.

That the posterior line of pits is an independent formation is, then, sufficiently well proved both by the ontogeny and the innervation. The arrangement and the number of the three head lines of pits are then essentially the same in *Polypterus* as in Palaeoniscids, Holosteans and Teleosts, to a certain degree also in Crossopterygians.

The *nasal line,* or, according to JARVIK, the "tectal" line of pits appears first in the 9.3 mm specimen in the shape of one single sense organ situated laterally to the first supraorbital sense organs (Fig. 7). Nothing excludes the possibility

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of the organ being a differentiation of the supraorbital line, as suggested by the writer (1947, p. 407).

The *mandibular lines of pits* do not appear until in the 9.3 and 9.6 mm specimens, probably owing to late development of the mandible itself. The anterior mandibular line (the anterior transverse portion of the mandibular line according to JARVIK, 1947) is in the 9.6 mm specimen formed by two sense organs, situated as a continuation rostrad of the mandibular canal line (Fig. 8). The number of sense organs is still the same in a 24 mm specimen.

The *vertical* and the *horizontal mandibular lines of pits,* each consisting in the 24 mm specimen of four neuromasts and by JARVIK called the posterior transverse and the longitudinal portion of the mandibular line, are not formed in the 6.75 mm specimen. In the 8 mm specimen as well as in the 9.3 and 9.6 mm specimens they are represented by two neuromasts lying close to the fourth mandibular canal organ (Figs. 3, 8). Each one is innervated by a special ramification of the ramus mandibularis. As in the 24 mm specimen the two lines in question are innervated in this way, there is no doubt that each organ represents a separate line, the most rostrally situated the primordium of the horizontal line, the posterior organ of the vertical line.

In the 6.75 mm specimen one single neuromast is situated rostrally to the first preopercular organ (Fig. 1). It is the primordium of the line the author (1947) called the primary mandibulary line, using the terminology of HOLMGREN (1942). JARVIK named it the *quadrato-jugal pit line,* a term also adopted in this paper. In the 8 mm specimen as well as in the 9.6 and 9.3 mm specimens the line is still formed by one sense organ (Figs. 3, 8). In the latter specimen it has moved further rostrad, in accordance with the incipient growth in length of the cheek. In the 24 mm specimen the number has increased to two organs.

The different *pit lines of the cheek,* are, in the 6.75 mm specimen, represented by only one neuromast (Fig. 1). It is situated caudally to the topmost postorbital organ. It is, then, the primordium of the anterior division of the horizontal pit line of the cheek. In the 8 mm specimen the number of sense organs between the infraorbital and the premandibular canal lines has increased to three (Fig. 3). The most rostral organ is, as in the younger specimen, the primordium of the anterior division of the horizontal line. The two posterior ones represent either the posterior division of the horizontal line, or the vertical line of the cheek, or both. In the 9.6 mm specimen there are still only the same three sense organs, one anterior, representing the anterior division, and two posterior organs (Fig. 8). In this specimen it has, however, been possible to trace the nerves of these two organs. They are each innervated by one separate branch of the hyoideo-mandibularis nerve, as are in the 24 mm specimen the horizontal and the vertical lines respectively. The most caudal organ should then represent the vertical line of pits, and the middle organ the posterior division

of the horizontal line. In the 24 mm specimen the former line has 2 sense organs, and the latter 4.

DEVELOPMENT OF DERMAL BONES

As might be expected no bony elements whatsoever are developed in the 6.75 mm specimen.

The 8 mm specimen has provided the solution of a much discussed problem concerning the bones in the cheek. As is well known (ALLIS 1905 and 1922, PEHRSON, 1922) the cheek in *Polypterus* is covered by a broad bone shield. In the posterior rim runs the preopercular canal with its three neuromasts. Various opinions concerning the homology of this bone have been expressed, but generally it is considered to be a preoperculum. This view is maintained, for instance, by HOLMGREN—STENSIÖ (1936), who also give a short summary of earlier views. JARVIK (1947) expresses the opinion that it has probably arisen through the fusion of elements at least in the main homologous with a squamosal and a preoperculum in Osteolepiform Crossopterygians. It should, then, be a complex squamoso-preopercular.

Judging from the conditions in a 24 mm specimen the author (1947) was inclined to consider the rostral and main portion of the bone to be developed originally as a process from the preopercular proper. At this stage (24 mm) the canal portion of the preopercular has the shape of an almost semilunar disk, formed in connection with the second and third neuromasts in the preopercular line. From the dorsal end of this disk protrudes, in a rostro-ventral direction, a long narrow process the rostral end of which partly overlaps a similar process from the maxillary, running in a dorsocaudal direction. It is the former process that later forms the main part of the broad cheek plate.

In the 8 mm specimen there are a few bones developed, yet none in connection with the sensory canals. The bones thus formed are all dermal: the maxillary, the dermo-palatine, the parasphenoid, and a short bone element dorso-caudally to the maxillary. In the mandible there are the dental sensu stricto, the angular sensu stricto, and the prearticular (Figs. 5, 6).

"MAXILLARY"

The *maxillary*, still without any teeth, has the shape of a lying sickle, the anterior end pointing rostro-dorsal and the posterior caudo-dorsal (Fig. 5).

Immediately behind the posterior end, which it slightly overlaps, is a short bone pointing in the same direction as the caudal half of the maxillary.

As to the interpretation of the homology of this bone the decisive fact is that it is apparently not connected with any sensory line. It lies far in front of the

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preopercular neuromasts, and no connection seems possible with the horizontal pit line either. Further, no latero-sensory elements are developed at this stage. If the possibility of the bone in question having a latero-sensory origin is ruled out, a circumstance that excludes the possibility of the bone being a "squamosal" element, there remains only one alternative, viz. an anamestic origin (the term used in Stensiö's broader sense). The question is, then, where a homology might be found. One possible element has already been suggested by STENSIÖ (1947), who points to the possibility of the rostral process from the preopercular representing a posterior portion of the maxillary proper. The author accepts this theory, in which case the bone at the posterior and of the maxillary in the 8 mm Polypterus is to be considered an ontogenetically independent portion of the maxillary proper.

In comparing the maxillary in *Polypterus* with the same bone in *Palaeoniscids*, two striking differences can be noted. One is that in *Polypterus* the anterior portion fuses with two infraorbital latero-sensory canal bones to form a complex maxillary-infraorbital. Another is the peculiar shape of the young maxillary forming a sickle-shaped rod. Earlier (1947) the author expressed the view that the posterior portion might correspond to a separate supra-maxillary, although at the stages then available no independent origin could be traced. That has not been the case at this early stage either. Instead a separate bone has developed dorso-caudally to the posterior process of the maxillary. To continue with the apparent differences between the maxillary in Palaeoniscids and in *Polypterus*, that bone in the former has a very broad posterior portion, absent in the latter. It might then be suggested that the missing portion partly corresponds to the ontogenetically appearing anamestic bone just mentioned. To this can be added also the anamestic bone in front of the quadrato-jugal (TRAQUAIR'S bone Y^1). Together they cover the space filled in Palaeoniscids by the mighty posterior portion of the maxillary.

As a matter of fact there are in Palaeoniscids indications of a subdivision of the posterior end of the maxillary. LEHMAN (1952) has described a specimen of Pteronisculus broughi (Fig. 9), in which this part of the maxillary does not form an unbroken line "mais entaillé par un certain nombre d'angles aigus formés par le bord antéroinférieur du préopercule; l'angle aigu superieur a son sommet sur le prolongement de la pit-line horizontale de la joue; aux autres angles, font suite des sutures que l'on peut suivre sur le maxillaire et qui découpent sur ce maxillaire des plaques distinctes à ornamentation différente de celle du reste de l'os." Lehman considers this to be "un exemple absolument incontestable de dissociation osseuse chez les Paléoniscides: 1° parce que les sutures sont nettes et sont prolongées par des émarginations du bord postérieure du maxillaire; 2° parce que les différents composants osseux ont une ornamentation différente; chaque composant devait donc avoir un centre de radiation indépendant". In another specimen of the same species Lehman has observed

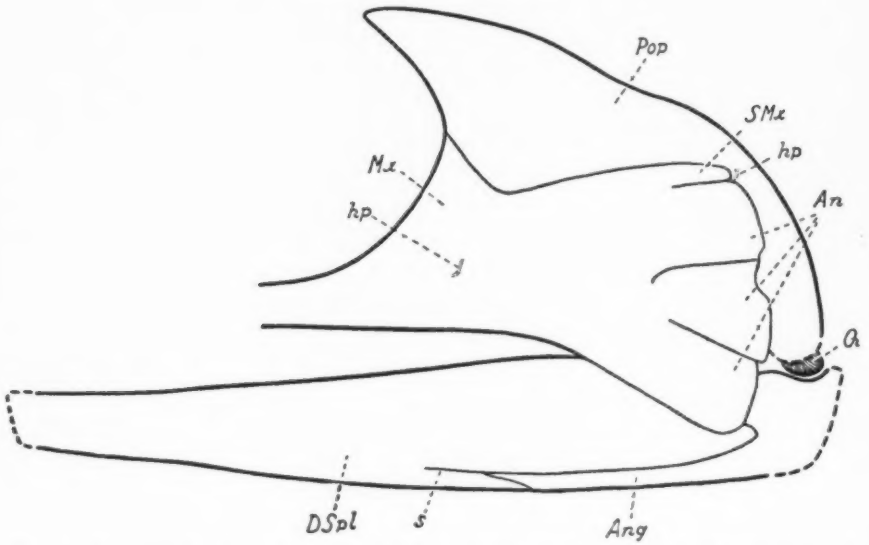

Fig. 9. *Pteronisculus broughi* (from LEHMAN, 1952). Part of skull in lateral view. *An* anamestic elements of maxillary, *Dspl* dentalo-splenial, *Pop* preopercular, *Q* condyle of quadrate, *Smx* supra-maxillary element of maxillary, *hp* horizontal pit line, *s* suture in dentalo-splenial. Other letters on p. 258.

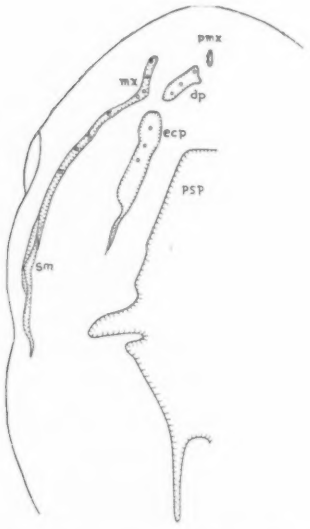

Fig. 10. *Polypterus*, 9.6 mm. Dermal skull in ventral reconstruction.

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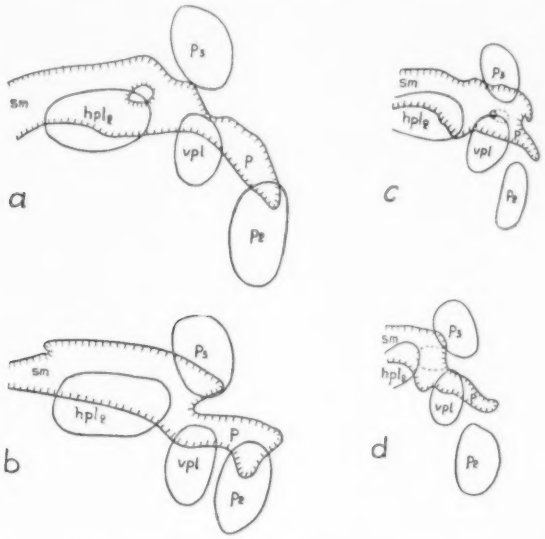

Fig. 11. *Polypterus*. Reconstruction of supramaxillary-preopercular in 9.6 mm specimen (*a* right, *b* left side) and in 9.3 mm specimen (*c* left, *d* right side).

that the posterior portion of the maxillary is incompletely divided into one large ventral and one smaller dorsal part, the latter being the homologue of the supramaxillary in the Holosteans.

It seems very probable, then, that the bone in question in the 8 mm *Polypterus* is also the homologue primordium of a supramaxillary element. If that is the case the maxillary in Polypterus shows a greater affinity to the palaeoniscid maxillary than has been supposed, and that in spite of other peculiarities.

Later, during the ontogeny, the *supramaxillary* element existing in the 8 mm specimen assumes an entirely different course. While the maxillary proper keeps its position the supramaxillary element lengthens in a dorso-caudal direction and fuses with the primordium of the preopercular.

"PREOPERCULAR"

The result of this development can be seen at the 9.6 mm stage (Fig. 8). As the figure shows, the supramaxillary has lost its independence. Instead there is formed a long process from the *preopercular,* situated in the position of the free bone at the earlier stage. The posterior end of the complex bone so formed can be studied in Fig. 11 a—d, representing the conditions on the right and the left side of the cheek in the 9.3 and the 9.6 mm specimens respectively.

The preopercular canal lodges three neuromasts. As already pointed out by the author (1947) the preopercular in the 24 mm specimen is developed as a

canal bone lodging the neuromasts Nos. 2 and 3, while a separate primordium is forming in connection with the neuromast No. 1. Whether the part of the bone surrounding the two dorsal neuromasts had originated as two separate primordia, each in connection with one sense organ, or whether it had formed as one single piece, was not possible to discern in the 24 mm specimen. Judging from the ontogeny of the other canal bones it seemed so far to be a general rule in *Polypterus* that a separate bone primordium forms in connection with each neuromast.

It cannot be definitely proved that that is the case concerning the dorsal portion of the preopercular, as an intermediate stage between the 8 mm and the 9.3 mm stage is missing. Yet, a study of the shape of the preopercular primordium in the 9.3 and 9.6 mm specimens seems to support the view that the caudal end of the preoperculum may actually consist—apart from the afore-mentioned supramaxillary element—also of two preopercular primordia, each one formed in connection with one neuromast.

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In the 9.3 specimen the bone on the left side (c) has a small foramen just between the two neuromasts, a foramen obviously on the way to being filled out. This is shown also by its being continued caudad by a very thin fenestration. This fenestra formation is also noticeable in the bone on the right side, as is indicated in the figure (d), even if there is nothing left of the foramen. In the 9.6 mm specimen the preoperculum primordium on the right side (a) also has a foramen, but on the left side it has instead a deep incision in the caudal edge (b). It seems beyond doubt that these conditions indicate the beginning of a fusion of separate elements.

As mentioned, the most ventral portion of the preopercular bone is just beginning to form as an independent primordium in the 24 mm specimen. Later this primordium fuses with the dorsal part earlier formed. The "preopercular" bone in *Polypterus* has, thus, originated from four elements, viz. three latero--sensory elements, each one arisen in connection with one neuromast, and further an anamestic bone component that might be called a supramaxillary. The bone is, then, a complex preopercular-supramaxillary. The other instances of that type of complex bones in *Polypterus* are the "premaxillary", the "maxillary", the "dentary", and the "angular".

Although the tendency to form complex bones by the fusion of latero-sensory and anamestic elements is extremely advanced in *Polypterus,* it is not unique. A complex rostro-premaxillary exists in Crossopterygians, as in most Palaeoniscids. Another manifestation of the same kind is the formation of a complex dental-splenial in most bony fishes.

As to this last-mentioned bone, it does not exist as such in early stages in *Polypterus.* In the 8 mm as well as in the 9.3 mm and 9.6 mm specimens there are no splenials developed (Figs. 5, 8).

"DENTARY"

In the two oldest specimens the *dentary* is developed as a tooth-bearing bone, but there is still no indication of latero-sensory elements. As shown by the author (1947) there are, however, three splenials developed in the 24 mm specimen in connection with the organs Nos. 1—3 in the mandibular series. These three splenials have formed in order from behind and rostrad. The first splenial is still an entirely free canal bone without any connection with the dentary proper. The second and third are already partly fused with that bone, although a longitudinal furrow between the dental and the second and third splenial shows where the fusion has taken place and the rostral end of the splenial No. 2 is still independent. In a 29 mm specimen all three splenials are firmly fused with the dentary, together with which bone they have formed the dentalo--splenial. The angular-splenial is formed in the same way. There can be no doubt that, at a stage between the 9.6 mm specimen and the 24 mm one, so far not found, the independent formation of the entire splenial series could be observed. The conditions in the larval Polypterus recall what is seen, for instance, in the Crossopterygian *Megalichthys*.

This circumstance may in any case not be called upon in order to prove more than a very general affinity to the Crossopterygians. Also in Amia (Pehrson, 1940) the splenials develop in a way that points to their earlier independent formation. To a certain degree that is also the case in a Teleostean like *Esox* (Pehrson, 1944). As adult these fishes do not show anything but a complete fusion of the dental and the latero-sensory elements, as is also the case in Palaeoniscids.

"PREMAXILLARY". DERMOPALATINE. ECTOPTERYGOID. PARASPHENOID

As to the other bones developing at these early stages of Polypterus, they do not offer anything of special interest, with the exception of the angular, a point which will be mentioned below. In the 9.3 and the 9.6 mm specimens there is formed a small *premaxillary* primordium on each side, each carrying three teeth (Figs 8, 10). A row of teeth has also developed on the maxillary, as is the case in the *dermopalatine*. A comparatively large *ectopterygoid* has developed, armed with some teeth. The *parasphenoid* has grown enormously in comparison with the poor appearance at the previous stage, and has almost entirely assumed the shape it has in a 24 mm specimen. Yet, no teeth are formed so far.

"ANGULAR"

The *angular* is formed in two separate elements (Fig. 8), a large anterior and a smaller posterior. The two canal organs, which are later going to form the

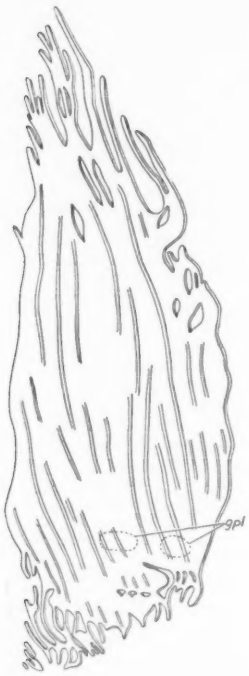

Fig. 12. *Polypterus*, 24 mm. Reconstruction of right gular primordium in ventral view.

angular splenials, are situated outside the rostral element, and so are the primordia of the horizontal and the vertical mandibular lines of pits. The caudal element of the angular is to be considered a surangular, which later fuses with the main angular element to form a complex angular-surangular, a bone considered by LEHMAN (1952) to be a normal constituent in the palaeoniscid mandible.

GULAR

The ontogeny of the gulars in Polypterus is not known as yet. L. S. BERG (1940) mentions that they represent branchiostegal rays. Anyhow nothing has so far been proved concerning their nature.

At the 9.6 mm stage there is still no indication of the gulars, although the gular line is formed and consists of two sense organs on each side. At the 24 mm stage, on the other hand, they are fairly well developed. As the reconstruction of a gular plate at this stage shows (Fig. 12), there seems to be no doubt that they actually are derived from branchiostegal rays. The middle part already forms one continuous shield of thin bone, whereas the rostral and the causal ends still show the origin from slender rays of bone. The surface of the shield-

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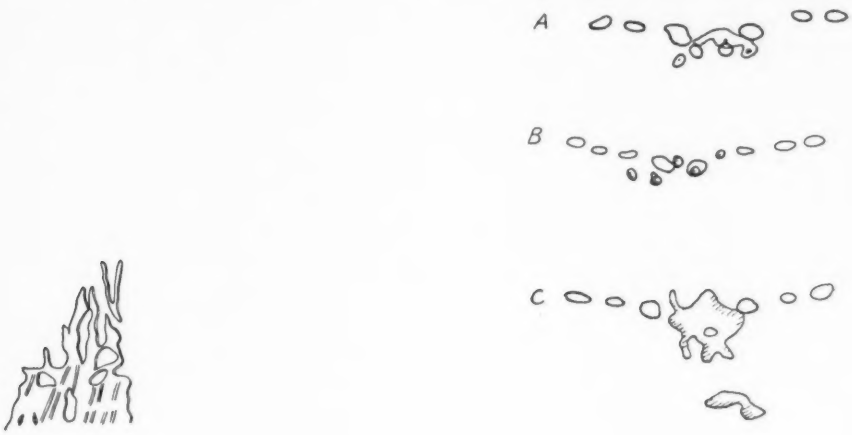

Fig. 13. *Polypterus*, 27 mm. Reconstruction of rostral end of right gular primordium in ventral view.

Fig. 14. *Amia calva*. Three different stages in the development of the gular bone. A, 12 mm specimen; B, 12.5 mm; C, 13 mm. (62 × 1.) Oblique lines, gular primordia; white circles, pit organs in the gular line. (PEHRSON, 1940.)

-like part has a distinct pattern of more or less parallel narrow ridges also indicating the origin from parallel rays. The two gular pit line organs are situated over the caudal part of the bone, as the figure shows.

Still in a 27 mm specimen (Fig. 13) the gulars grow by means of the addition of ray-like elements.

The development of the gular plates in *Polypterus* also provides the clue to an understanding of the origin of the gulars i *Amia*. The author has described their ontogeny in an earlier paper (PEHRSON, 1940). The first appearance of a primordium takes place in this fish in a 12.5 mm specimen, as a transversal line of separate rudiments (Fig. 14) in the immediate vicinity of the gular pit line. Later the primordia fuse to form a small plate which grows in all directions. It seems probable that the small dot-like primordia in *Amia* represent branchiostegal rays only in a considerably reduced state.

PHYLOGENETICAL REMARKS

The new contributions to the understanding of the systematic position of *Polypterus* added by this investigation, do not seem to alter the opinion earlier maintained by the author that this fish is, in the main, more closely related to the Palaeoniscids than to any other group of fishes. In any case they do not seem to support the theory of closer affinities to the Crossopterygians. The

characters in *Polypterus* pointing in the direction of relationship to the Crossopterygians seem to be so generalized—for instance the structure of the mandible—that they appear in Holosteans like Amia and even higher amongst the Actinopterygians. Nor does the ontogeny of the pit line system appear to recall conditions in the Crossopterygians.

Apart from the characters recalling conditions in the Palaeoniscids, *Polypterus* is endowed with a series of specialities in so far as the structure of the dermal skull is concerned which makes it impossible to place it amongst the Palaeoniscids sensu stricto. The most striking of these specialities is the tendency to form complex bones by means of fusion of anamestic bones and latero-sensory elements. Another is the ontogenetical appearance of spurious parietals, which after having vanished are replaced by the supratemporo-intertemporals (PEHRSON, 1947).

The origin of the various dermal bone elements and their fate in the adult is demonstrated in the table below. The author has also presented there the results obtained in the earlier investigation often referred to in this paper (PEHRSON, 1947).

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LATERO-SENSORY CANAL ORGANS AND BONES ORIGINATING IN CONNECTION WITH THEM IN POLYPTERUS

Canal organs	*Separately developing canal bones*	*Definite bones*
supraorbital 1	nasal 1	nasal 1 (acessory nasal)
supraorbital 2	nasal 2	nasal 2
supraorbital 3	nasal 3	nasal 3
supraorbital 4	frontal element 1	frontal
supraorbital 5	frontal element 2	
supraorbital 6	frontal element 3	
infraorbital 1	rostral	rostral
infraorbital 2	lateral rostral	+ premaxillary = "premaxillary"
infraorbital 3	antorbital 1	
infraorbital 4	antorbital 2	
infraorbital 5	lacrymal	lacrymal
infraorbital 6	infraorbital 1	+ maxillary = "maxillary"
infraorbital 7	infraorbital 2	
infraorbital 8	postorbital	postorbital
infraorbital 9	dermosphenotic	dermosphenotic
infraorbital 10	intertemporal	supratemporo-intertemporal (= "parietal")
postotic	supratemporal	
supratemporal crosscommissure 1	extrascapular 1	extrascapular 1
supratemporal crosscommissure 2	extrascapular 2	extrascapular 2
supratemporal crosscommissure 3	extrascapular 3	extrascapular 3
mandibular 1	splenial 1	+ dentary = "dentary"
mandibular 2	splenial 2	
mandibular 3	splenial 3	

mandibular 4	splenial 4	+ angular + surangular =
mandibular 5	splenial 5	= "angular"
preopercular 1	preopercular 1	+ supramaxillary = "pre-
preopercular 2	preopercular 2	
preopercular 3	preopercular 3	opercular"

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KEY TO THE FIGURES

an anterior nasal aperture
ang angular
apl anterior pit line
ch notochord
co cement organ
de dental
dp dermoplatine
dsp dermoshpenotic sense organ
e eye
gpl gular pit line
hm hyomandibular
hmpl horizontal mandibular pit line
hpl horizontal pit line
hpl$_1$ horizontal pit line, anterior division
hpl$_2$ horizontal pit line, posterior division
i$_1$—*i*$_8$ infraorbital sense organs
ito intertemporal sense organ
m$_1$—*m*$_3$ mandibular sense organs
mc Meckelin cartilage
mpl middle pit line
mx maxillary
na nasal aperture
npl nasal pit line
p preopercular
pq palato-quadrate
p$_1$—*p*$_3$ precular sense organs
pn posterior nasal aperture
ppl posterior pit line
pra preaticular
ps parashpenoid
qjl quadrato-jugal pit line
s$_1$—*s*$_6$ supraorbital sense organs
sang supraangular
sco supratemporal commissure sense organ
sm supramaxillary bone
sp spiracle
ssco suprascapular sense organ
sto supratemporal sense organ
vmpl vertical mandibular pit line
vpl vertical pit line of the cheek

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ON SEASONAL VARIATIONS IN THE PRODUCTION OF MALE SPERM ANTAGGLUTIN AND ITS RELATION TO FERTILITY IN BULLS AND RABBITS

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In 1952 LINDAHL and KIHLSTRÖM demonstated the occurence of factors, capable of counteracting and preventing the head-to-head agglutination of spermatozoa in the genital secretions of either sex. Agglutinated spermatozoa show reduced motility and at artificial insemination low fertility (MILOVANOV, quoted from ANDERSSON 1945 a, p. 53). Thus the two anti-agglutinic factors, named male and female sperm antagglutin, respectively, seem to be indispensable in the fertilization process, and some observations indicate a close relationship between fertility and the existence of active antagglutin in the male secretions. Thus preparations from bull semen during the summer, when fertility is high, generally gives a greater amount of antagglutin than in winter and early spring, when the fertility is lower. Further, in a number of subfertile and sterile individuals disturbances in the production of active antagglutin could be demonstrated (LINDAHL 1956). Consequently it seemed justified to study the seasonal and individual variations in the production of male sperm antagglutin and its relation to fertility in bulls and rabbits.

MATERIAL AND METHODS

Throughout the year semen has been collected regulary twice a week from twelve albino rabbits by means of an artificial vagina. By analysis of the

samples obtained the seasonal variations in some activities of the accessory glands, especially the production of male sperm antagglutin, have been studied.

By courtesy of the Artificial Insemination Association at Eskilstuna and its chief veterinarian, Dr. B. Ström, it has been possible to study all ejaculates from 32 bulls (Swedish Red Breed), owned by this Association from Jan. 5, 1956 to Jan. 4, 1957. At the bull station the quality of all ejaculates has been assessed according to ordinary veterinary routine by measuring their volumes and by observing density, mass-activity, and motility. Density and mass-activity were subjectively estimated according to a three-graded scale, motility according to a four-graded scale. Besides the veterinaries have noted down other observations worthy of notice, such as agglutination of the sperm cells, abnormal sexual behaviour of the bulls, etc. With a few exceptions the bulls have been put to service regularly every seventh day, and generally two, or in a few cases three ejaculates have been collected from the same bull within thirty minutes. From each ejaculate a sample, 0.2—0.5 ml, was taken, rapidly frozen (KIHLSTRÖM 1956, 1958 a), and stored in a refrigerator until analysed.

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All samples have been analysed as to their contents of antagglutin according to LINDAHL, KIHLSTRÖM, and ROSS (1957) and KIHLSTRÖM (1958 a) with two slight modifications. After salting out the antagglutin twice, and dissolving the precipitate in distilled water the samples are evaporated at 20° C under vacuum over phosphorpentoxide. Secondly the centrifuge tubes used at extraction are equipped with a thick bottom. This makes it possible to powder the heated products with a glass pestle, and thus to perform all operations in the same recipient. In most cases 0.2 ml of the seminal fluid have been used at analysis in order to get more reliable figures. Moreover in large enough samples the percentage of solids has been determined as described earlier (KIHLSTRÖM 1958 b). In some cases the sperm plasm has been analysed for its content of fructose according to the method worked out by ROE (1934), and modified by GROPPER (1954).

TREATMENT OF THE DATA OBTAINED

If considered of good quality the ejaculates yielded by the same bull on the same occasion were pooled before use for artificial insemination. The fertilities of such pools of ejaculates have been calculated as pregnancies in per cent of performed first inseminations. The results of the inseminations carried out at the Artificial Insemination Association at Eskilstuna and at its three branches at Strängnäs, Malmköping, and Stora Sundby have been compared by calculating the fertility of the individual bulls from more than 200 inseminations at each insemination station. The figures obtained show that at branch number one (See Table I) the results of the inseminations are significantly lower than

TABLE I

Mean fertility obtained at different insemination stations.

	Insemination stations			
	Eskilstuna	Branch no.		
		I	II	III
Fertility, %	60.4 ∓ 0.88	57.8 ∓ 0.83	62.1 ∓ 1.00	60.4 ∓ 0.83

at the other stations. The inseminations performed at this branch have thus been excluded, when calculating the fertilities of the pooled ejaculates.

ERB and co-workers (1950) have studied the number of inseminations necessary to get a reliable estimate of the fertilizing capacity of a single semen sample. For securing a dependable result they recommend 300 first and second inseminations. The figures given in their paper show, however, that the coefficient of variability changes only slightly, when the number of inseminations increases from 20 to 300, whereas this coefficient shows very high values, when the number of inseminations is below 20. Besides, they have studied the correlation between the non-return rate for inseminations performed at the day of collection and that of inseminations performed the next day with the same sample of semen. At 22 or more inseminations per day they found a highly significant correlation. These facts indicate that about 20 or more inseminations per sample would give sufficiently reliable results. It must also be kept in mind that ERB and co-workers have calculated their figures on the basis of 60 days non-return rate after first and second inseminations, whereas the present writer has calculated fertility as prooved pregnancies in per cent of first inseminations performed, thus reducing the variability of the figures obtained. The accuracy of this index of fertility has been tested by calculating the standard deviation for two groups of figures according to the number of cows inseminated, (group 1, 20 and less inseminations, $s = 17.773$, group 2, more than 20 inseminations, $s = 2.672$). Consequently only bulls, the semen of which has been used for inseminations of more than 20 cows at each semen collection can be included in the investigation. Furthermore it soon became obvious that the relationships between production of antagglutin and fertility are subjected to wide seasonal variations. Reliable results could thus be based only upon figures obtained from bulls which had been used continuously throughout the year without longer pauses. Seven bulls fullfilled these two conditions, and have been subjected to a more detailed study. Moreover one bull which often shows spontaneous agglutination of the sperm cells has been studied more intensely. Sometimes agglutination was observed also in semen from other bulls. When such ejaculates have been used at artificial inseminations they have been separated from the normal ones at the statistical treatment.

The figures obtained at the analyses have been studied in relation to fertility using conventional statistical methods, mainly according to HALD (1948a and b). The abbreviations r, R, and b are used for the correlation coefficient, the multiple correlation coefficient, and the regression coefficient, respectively.

RESULTS AND DISCUSSION

a) *The seasonal variations in the production of male sperm antagglutin in rabbits.*

In rabbits the seasonal variations in the activity of the sexual glands are very pronounced. The ejaculated volumes as well as the amounts of antagglutin

TABLE II

Mean values of semen characteristics obtained during one year from twelve rabbits.

Month	Vol. ml.	μg of antagglutin per ejaculate	μg of antagglutin per ml.	Solids mg per ejaculate	%
Jan.	0.72	6.08	9.58	34.7	3.35
Feb.	0.77	4.30	6.36	32.1	4.46
March	0.62	4.06	6.36	30.4	4.36
April	0.69	4.64	6.42	23.6	3.42
May	0.51	3.37	5.92	18.1	3.35
June	0.44	5.67	12.99	13.5	3.08
July	0.52	5.20	9.27	23.6	3.57
Aug.	0.47	2.84	5.69	20.4	3.69
Sept.	0.50	2.52	5.31	20.3	3.21
Oct.	0.50	5.89	8.01	21.4	3.71
Nov.	0.61	7.19	10.39	23.6	4.51
Dec.	0.82	9.37	11.59	44.2	4.77
The year	0.59	4.72	7.74	24.3	3.72

Comparisons between mean values.

Months	Volume		μg of antagglutin per ejaculate		μg of antagglutin per ml		Solids mg per ejaculate		%	
	t	p <	t	p <	t	p <	t	p <	t	p <
Dec.—May	4.07	0.005	3.22	0.005	2.95	0.005	2.82	0.005	2.85	0.005
Dec.—Aug.	4.85	0.0005	3.50	0.0005	2.98	0.005	2.50	0.01	2.01	0.05
Dec.—Sept.	4.48	0.0005	3.70	0.0005	3.15	0.005	2.60	0.01	2.41	0.025
July—June	1.60	0.01	—	—	—	—	4.00	0.005	—	—
July—Aug.	1.25	0.2	—	—	—	—	0.96	0.2		
Aug.—June	—	—	—	—	—	—	—	—	1.78	0.05
Aug.—Sept.	—	—	—	—	—	—	—	—	0.95	0.2
June—May	—	—	1.78	0.05	3.03	0.005	—	—	—	—
June—Sept.	—	—	2.59	0.01	3.21	0.005	—	—	—	—

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and of total solids show maximal values from November to February and a secondary maximum in the middle of the summer (See Table II and Fig. 1). Low values are found during May—June and August—September, and for all properties studied the values obtained during these months differ significantly from those determined during the maximum in December (See Table II). The maximum during summer is less pronounced, and the variations of the individual properties are not quite synchronous. The volumes and the total amounts of solids have their maximum in July, whereas the percentage of solids is greatest in August. This secondary maximum is most striking for the production of antagglutin which is greatest during June.

According to MACIRONE and WALTON (1938) in the rabbit the number of spermatozoa per ejaculate is lowest in early autumn, while FRÖLICH (1948) found a low concentration of spermatozoa in semen ejaculated during autumn and winter. Also the initial sperm motility and its duration at storage are lowest during July and August (FRÖLICH 1948). It is interesting to compare these facts with the experience of practical breeders, viz. that rabbits show low sexual activity while moulting. This is in agreement with the findings by FRÖLICH (1948 and 1950) who noticed that only few female rabbits were willing to copulate from the middle of July to the end of December, and that only a fraction of these became pregnant. When collecting semen by means of an artificial vagina the present writer has found that also male rabbits are comparatively unwilling to copulate in late spring and early autumn. Thus the low productivity of the sexual glands seems to coincide with a feeble libido.

Many factors may be important in regulating the activity of the sexual glands. The increasing daylight during spring and early summer might have a stimulating effect. The present writer (1956, 1958 a and b) has shown that in rabbits treatment with artificial light during the winter invigorates the activity of the accessory glands, and that there is a lag of about two months from the beginning of the treatment until the effect can be observed. The supply of fresh food during summer may also act stimulating.

b) *The seasonal variations in the production of male sperm antagglutin in bulls.*

In all individual bulls the production of antagglutin is highest in late summer and early autumn, and at a minimum in late winter. The monthly mean amounts of antagglutin per ejaculate of the seven selected bulls are given in Table III and Fig. 2. Also the ejaculated volumes and the amounts of total solids per ejaculate reveal a similar variation according to season. Thus we may conclude that the sexual glands are most active during late summer and early autumn. This high activity is reflected in the properties of the semen as well as in the fertility of the bulls. These synchronous variations may, however, give a false picture since fertility depends also upon the sexual condition of the females.

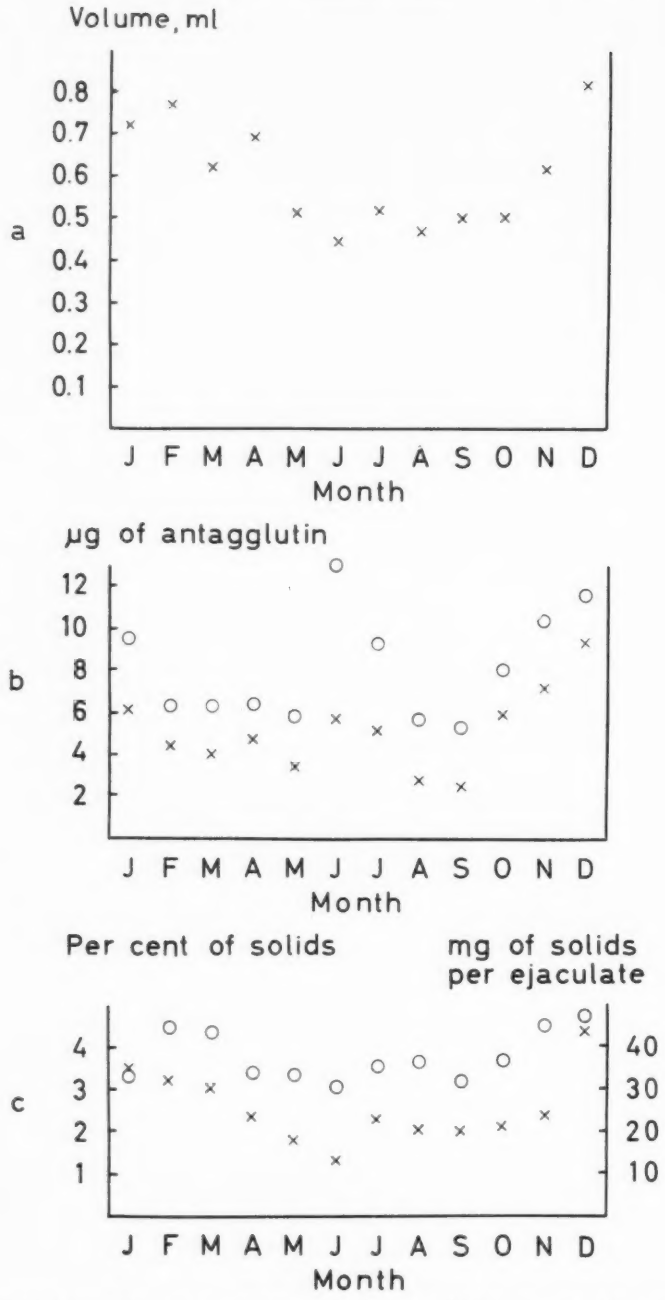

Fig. 1. Seasonal variations of some characteristics of rabbit semen. a) Mean volumes per month. b) Mean amounts of antagglutin per month. (× = µg per ejaculate, ⊙ = µg per ml). c) Mean amounts of solids per month. (× = mg per ejaculate, ⊙ = per cent).

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TABLE III

Mean values of semen characteristics obtained during one year from seven selected bulls. In brackets mean values from 32 normal bulls

Month	Vol. ml.	μg of antagglutin per ejaculate	μg of antagglutin per ml.	Solids mg per ejaculate	Solids per cent	Fertility, per cent
Jan.	5.05 (4.61)	21.92 (19.00)	4.38 (3.81)	487.3 (470.5)	9.47 (9.41)	58.3 (57.9)
Feb.	4.30 (4.43)	19.25 (16.90)	4.45 (3.85)	416.4 (414.3)	9.20 (9.11)	57.2 (57.4)
March	4.45 (4.62)	20.52 (19.04)	4.38 (4.07)	479.2 (448.0)	9.82 (9.50)	57.7 (58.0)
April	4.66 (4.47)	21.90 (21.16)	4.67 (4.51)	474.0 (471.7)	10.08 (10.04)	59.8 (57.4)
May	4.69 (4.78)	27.96 (27.82)	5.92 (5.88)	493.0 (498.0)	10.26 (10.28)	60.1 (59.8)
June	5.35 (4.94)	29.50 (27.96)	5.56 (5.38)	588.5 (575.4)	10.65 (11.12)	63.1 (63.7)
July	5.11 (5.30)	30.02 (27.70)	5.72 (5.28)	602.5 (598.3)	11.90 (11.69)	65.6 (64.4)
Aug.	5.50 (5.07)	34.49 (33.48)	6.41 (6.20)	639.6 (632.5)	11.61 (11.42)	64.4 (64.1)
Sept.	5.10 (5.22)	31.20 (31.20)	6.19 (6.10)	597.4 (597.5)	11.52 (11.52)	68.2 (68.2)
Oct.	5.10 (5.17)	29.52 (29.36)	6.28 (6.28)	542.5 (538.1)	10.83 (10.82)	67.5 (64.6)
Nov.	5.67 (5.25)	31.00 (31.10)	5.57 (5.54)	688.0 (688.5)	12.18 (12.08)	65.5 (63.7)
Dec.	4.92 (4.71)	26.31 (26.00)	5.50 (5.48)	511.5 (506.2)	10.15 (10.18)	64.3 (64.3)
The year	4.99 (4.88)	26.75 (25.17)	5.38 (5.07)	540.0 (532.8)	10.61 (10.57)	62.0 (61.5)

Comparison between mean values

Months	Volume		μg of antagglutin per ejaculate		μg of antagglutin per ml.		Solids mg per ejaculate		Solids per cent		Fertility	
	t	p	t	p	t	p	t	p	t	p	t	p
Aug.-Febr.	7.32	< 0.0005	4.25	< 0.0005	3.16	< 0.005	5.50	< 0.0005	6.27	< 0.0005	2.06	< 0.025
Sept.-Febr.	2.98	< 0.005	3.60	< 0.0005	2.53	< 0.01	3.70	< 0.005	5.48	< 0.0005	3.69	< 0.0005

Many investigations have been devoted to the study of seasonal variations in fertility. There exist, however, in this respect great differences between different breeds and climates (Cf. e.g. ANDERSSON 1945 b, 1948 and MERCIER and SALISBURY 1946). Danish cattle have the lowest fertility during May and June, and the highest during October (ROTTENSTEN 1955). According to SWENSSON (1952) the fertility of the Swedish Red Breed is at a minimum in February and at a maximum during September. This is in agreement with the results of the present investigation. All mean values of the properties studied obtained during August and September are significantly higher than those referring to February (See Table III).

The increasing daylight during spring and early summer may stimulate the activity of the sexual glands. MERCIER and SALISBURY (1946) have shown that in their material from eastern Canada the fertility level of the bulls was significantly correlated to the length of daylight, there being a lag of one to two months before the effect of the increase of light reached its maximum. These findings are in agreement with the results obtained by the present writer (1956, 1958 a and b) from the treatment of rabbits with artificial light during winter. The free supply of fresh food during summer may also be an important factor for the activation of the sexual glands.

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c) *The relationship between some characteristics of the semen and the fertility of the same sample.*

In this respect the bulls show very wide individual variations, and there exist also great seasonal variations. One bull, 104 Bergshamra (code number 434), shows throughout the year a significant correlation between fertility and the content of antagglutin in the semen (See Table IV). Besides there is also a highly significant correlation between fertility and the mean percentage of solids in the pooled ejaculates used for inseminations. No multiple correlation between these factors could, however, be demonstrated, the multiple correlation coefficient, R, being very low, 0.2254. In the semen from this bull no other properties studied show any relation to fertility (See Table IV).

In other bulls the correlation between the production of antagglutin and the fertility is limited to certain periods. Three bulls, 13 Frörum (code number 426), 65 Trantorp (code number 431), and 406 Boxholm (code number 450), exhibit correlations between fertility and the content of antagglutin during the early part of the year (See Table IV). In the semen of bull number 13 Frörum no other characteristics studied are correlated to fertility, whereas the mean volumes of the ejaculates yielded by bull number 406 Boxholm are correlated to fertility throughout the year. The correlation between fertility and the content of antagglutin in the semen of bull number 65 Trantorp ceases in the beginning of April, and is succeeded in the middle of April by a significant

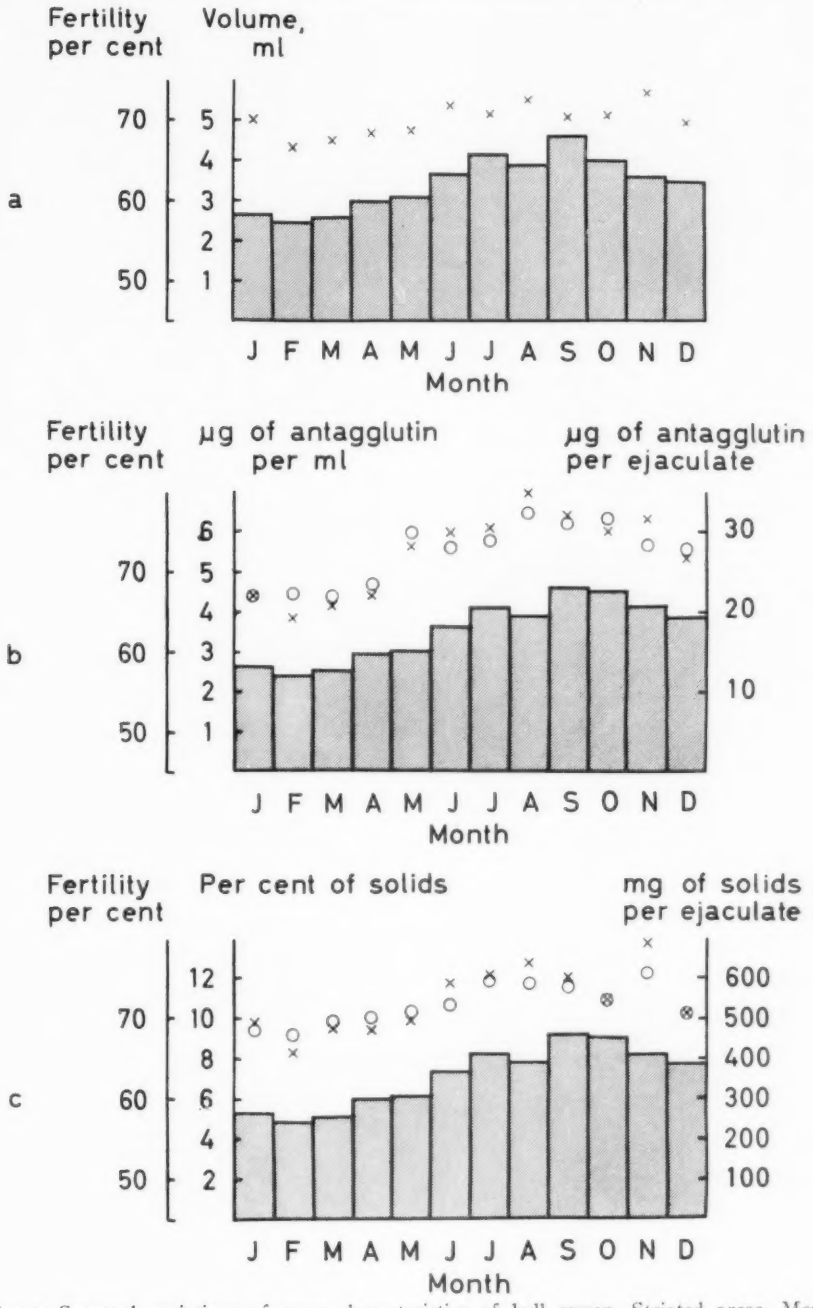

Fig. 2. Seasonal variations of some characteristics of bull semen. Striated areas, Mean fertility in per cent. a) Mean volumes per month. b) Mean amounts of antagglutin per month. (× = µg per ejaculate, ⊙ = µg per ml). c) Mean amounts of solids per month. (× = mg per ejaculate, ⊙ = per cent).

TABLE IV

Correlations between properties of semen and fertility in seven selected bulls.

Bull, code no.	Mean fertility of the bull	Properties of the semen in the pooled ejaculates correlated to fertility									
		μg of antagglutin per ejaculate	Period	μg of antagglutin per ml	Period	μg of antagglutin per pool	Period	Per cent of solids in the semen	Period	Mean volume per ejaculate	Period
418 (10 years old)	60.1	No correlation		No correlation		No correlation		$r = 0.5910$ $v^2 = 9.12$ $p < 0.01$ $t = 3.0208$ $p < 0.005$	5/1 -56 — 4/1 -57	No correlation	
426 (8 years old)	65.3	$r = 0.5560$ $v^2 = 35.84$ $p < 0.0005$ $t = 1.8916$ $p < 0.05$	16/1 -56 — 30/4 -57	No correlation		No correlation		No correlation		No correlation	
431 (7 years old)	63.5	$r = 0.9950$ $v^2 = 29.33$ $p < 0.01$ $t = 5.4546$ $p < 0.025$	22/2 -56 — 7/4 -56	$r = 0.9680$ $v^2 = 186.00$ $p < 0.01$ $t = 14.07$ $p < 0.005$	22/2 -56 — 7/4 -56	No correlation		$r = 0.6012$ $v^2 = 5.67$ $p < 0.05$ $t = 2.3789$ $p < 0.025$	17/4 -56 — 4/1 -57	No correlation	
434 (6 years old)	63.9	$r = 0.4518$ $v^2 = 6.43$ $p < 0.025$ $t = 2.5321$ $p < 0.01$	5/1 56 — 4/1 -57	$r = 0.5478$ $v^2 = 10.73$ $p < 0.005$ $t = 3.2612$ $p < 0.005$	5/1 -56 — 4/1 -57	$r = 0.4773$ $v^2 = 7.38$ $p < 0.025$ $t = 2.7206$ $p < 0.01$	5/1 -56 — 4/1 -57	$r = 0.6937$ $v^2 = 20.38$ $p < 0.0005$ $t = 4.5173$ $p < 0.0005$	5/1 -56 — 4/1 -57	No correlation	

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446 (3 years old)	61.9	$r = 0.8489$ $v^2 = 15.59$ $p < 0.01$ $t = 3.9494$ $p < 0.005$	13/8 -56 — 4/12 -56	$r = 0.8151$ $v^2 = 5.95$ $p < 0.1$ $t = 2.4370$ $p < 0.05$	13/2 -56 — 8/6 -56	No correlation		No correlation		No correlation	
450 (4 years old)	62.2	$r = 0.8850$ $v^2 = 18.13$ $p < 0.01$ $t = 4.2499$ $p < 0.005$	14/3 -56 — 9/5 -56	No correlation		No correlation		No correlation		$r = 0.5237$ $v^2 = 10.85$ $p < 0.005$ $t = 3.4741$ $p < 0.001$	5/1 -56 — 4/1 -57
451 (3 years old)	59.7	No correlation		No correlation		No correlation		$r = 0.7520$ $v^2 = 19.50$ $p < 0.005$ $t = 4.4183$ $p < 0.0005$	5/1 -56 — 4/1 -57	$r = 0.3389$ $v^2 = 4.53$ $p < 0.05$ $t = 2.1310$ $p < 0.025$	5/1 -56 — 4/1 -57

Formula used: $$v^2 = \frac{\left[\Sigma (x - \bar{x})(y - \bar{y})\right]^2 (n - 2)}{\Sigma (x - x)^2 \left[\Sigma (y - \bar{y})^2 - \frac{\left[(x - \bar{x})(y - \bar{y})\right]^2}{\Sigma (x - \bar{x})^2}\right]}$$

$$t = r \sqrt{\frac{n - 2}{1 - r^2}}$$

correlation between fertility and percentage of solids in the seminal fluid (See Table IV). Bull number 413 Boxholm (code number 446) shows a distinct relationship between fertility and content of antagglutin from the middle of August to early in December, and a tendency towards correlation during spring and early summer (See Table IV). There are, however, no correlations to any other property of semen studied during other parts of the year.

Finally two bulls, 593 Bjärka Säby (code number 418) and 214 Biby (code number 451), do not show any correlations at all between fertility and the content of antagglutin in the semen (See Table IV). In both cases there exist, however, highly significant correlations between fertility and the mean percentage of solids in the pooled ejaculates inseminated (See Table IV). Besides, bull number 214 Biby (code number 451) shows a feeble correlation between fertility and the mean volume of the pooled ejaculates. It is striking that the two last mentioned bulls, lacking correlation between fertility and the content of antagglutin, have the lowest fertility of the bulls studied. During the period of investigation the semen of these two bulls has been used for a total of 3288 inseminations, 1970 of which resulted in pregnancies, which gives a mean fertility of 59.9 per cent. The semen of the five remaining bulls has been used for 9306 inseminations of which 5909 resulted in pregnancies, giving a mean fertility of 63.5 per cent. The mean fertilities of these two groups of bulls have been compared using χ^2-analysis for heterogeneity, revealing a highly significant difference ($\chi^2 = 17.96$, $p < 0.0005$). In the production of antagglutin, however, these two groups of bulls show no significant differences.

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d) *The contents of antagglutin in abnormal ejaculates.*

In five ejaculates from normal bulls and in most ejaculates from the bull 6 Sör-Tensta (code number 439) the spermatozoa show a more or less pronounced agglutination. These ejaculates have been studied separately and compared with the same number of control ejaculates selected at random from 1500 normal ejaculates. The analyses show (Table V) that the contents of antagglutin are nearly identical in the two groups of ejaculates, whereas the ejaculated volumes and the contents of solids and of fructose are higher in the control ejaculates. These findings indicate that the agglutination is not primarily dependent upon a lack of antagglutin. A comparison with other investigations (LINDAHL 1956) makes it probable that in these cases the antagglutin occurs in the inactive oxidized form. In spite of the apparently normal production of antagglutin the figures show that other functions of the accessory glands are weakened. These facts suggest that also the production of reducing principles in the semen may be diminshed, thus augmenting a more rapid oxidization and thereby inactivation of the antagglutin.

On account of their low quality according to the ordinary veternary esti-

TABLE V

Mean values of semen characteristics obtained from ejaculates showing agglutination of the sperm cells as compared with the same characteristics obtained from normal ejaculates.

	Vol. ml	μg of antagglutin per ejaculate	μg of antagglutin per ml	Solids mg per ejaculate	Solids per cent	Fructose mg per ejaculate	Fructose per cent
Ejaculates with gglutination	4.61 ± 0.316	31.79 ± 3.811	6.51 ± 0.447	427.6 ± 38.60	9.53 ± 0.245	8.90 ± 1.049	0.20 ± 0.012
Normal ejaculates	5.34 ± 0.458	31.87 ± 3.696	6.37 ± 0.640	572.0 ± 61.48	10.78 ± 0.346	9.39 ± 0.872	0.18 ± 0.012

mation a number of ejaculates have not been used for insemination. Volume and contents of antagglutin and of total solids of these ejaculates have been compared with the same values of an equal number of apparently normal ejaculates from the same bulls. For control to a given rejected ejaculate the corresponding ejaculate yielded at the nearest preceding day of semen collection was chosen. The figures thus obtained are recorded in Table VI under the headings of "rejected ejaculates" and "control ejaculates". The latter show higher values, but there are no significant differences between these two groups. It is, however, possible that sexual disturbances, manifested by defective ejaculates, are dependent upon processes acting for long time and thus influencing also the ejaculates selected as controls. For this reason these two groups of ejaculates were compared with the normal ejaculates selected at random and referred to above. The figures obtained from the latter ejaculates are recorded in Table VI under the heading "normal ejaculates". All characteristics studied show higher values in the normal ejaculates, but there are no

TABLE VI

Mean values of semen characteristics obtained from 53 rejected ejaculates and the 53 ejaculates in time nearest preceeding those rejected (control ejaculates) compared with the characteristics of normal ejaculates selected at random.

	Volume, ml	μg of antagglutin per ejaculate	μg of antagglutin per ml	Solids mg per ejaculate	Solids per cent
Rejected ejaculates	4.17 ∓ 1.857	21.14 ∓ 12.816	5.35 ∓ 0.529	442.3 ∓ 27.63	10.49 ∓ 5.690
Control ejaculates	4.72 ∓ 0.224	23.31 ∓ 3.789	4.74 ∓ 0.447	483.3 ∓ 86.70	10.35 ∓ 0.316
Normal ejaculates	5.34 ∓ 0.458	31.87 ∓ 3.696	6.37 ∓ 0.640	572.0 ∓ 61.48	10.78 ∓ 0.346

significant differences between these and those rejected. It is, however, worthy of notice that the values obtained from the rejected ejaculates show a much greater variability, suggestive of some possible disturbances in the functions of the accessory glands.

In most cases only one ejaculate has been rejected in a series of two or three ejaculates yielded on the same day, the other ejaculates being pooled and used for inseminations. The latter ejaculates have been studied together with those referred to above as "control ejaculates", and their average fertility (58.5 %) compared with that of the remaining ejaculates (63.0), using χ^2-test for heterogeneity. The figures show a weakly significant difference ($\chi^2 = 5.15$, $p < 0.025$) suggesting that ejaculates of low quality may indicate lasting disturbances in the sexual functions of the bull.

e) *The relationships between the content of antagglutin and some other characteristics of bull semen.*

The production of antagglutin in the prostate can be expected to run parallel to the production of total solids in the accessory glands, and in all bulls highly significant correlations between the amount of solids and that of antagglutin per ejaculate are found. The regression coefficients, b, calculated from the figures obtained from the individual bulls are nearly identical, ranging from 0.034 to 0.039. It is thus possible to conjoin the figures from all bulls, which gives a regression coefficient of 0.0359 and a highly significant correlation ($r = 0.4416$, $v^2 = 182.25$, $p < 0.0005$).

A priori it might appear probable that the production of antagglutin is regulated by the circulating amounts of testosterone in the body. The correlations demonstrated above and earlier results (KIHLSTRÖM 1958 c) may be looked upon as supporting this assumption. Nevertheless the present investigation does not reveal any correlation between the production of fructose, which is known to be regulated by testosterone (MANN 1954), and the production of antagglutin.

In assessing the quality of semen the veterinaries have classified two properties of semen, viz. the density of sperm cells and the mass-activity (wave-motion) according to a three-graded scale and the motility according to a four-graded scale. In order to study possibly occurring relationships between these properties and the production of antagglutin the contents of the latter in ejaculates ranking highest from one of these aspects have been compared with that of ejaculates of lower quality. Mass-activity seems to be most intimately connected with the production of antagglutin. The ejaculates showing the highest mass-activity demonstrate a significantly higher average amount of antagglutin per ejaculate than those inferior in this respect ($t = 5.00$, $f = 496$, $p < 0.0005$). Different motilities of the sperm cells, on the other hand, are not connected with different levels of antagglutin in the semen. When comparing ejaculates

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having different densities of sperm cells we must finally distinguish between the two groups of bulls mentioned above (p. 270), and here again the two bulls having the lowest fertility (code numbers 418 and 451) differ from the remaining animals. In the semen from these two bulls the average amount of antagglutin is significantly greater in ejaculates with the highest density of sperm cells (grade 4) than in ejaculates of lower density ($t = 2.177$, $f = 155$, $p < 0.025$). In the remaining bulls there are no such relationships.

As demonstrated above there exists throughout the year a good agreement between the mean level of fertility on the one hand and the mean values of the properties studied (content of antagglutin, content of total solids, ejaculated volume) on the other. This fact clearly demonstrates that these properties of the ejaculates are regulated by the sexual status of the individual, and thus supply information about the fertilizing ability of the semen. It must, however, be kept in mind that the figures referred to above are mean values based upon analyses of about 100 ejaculates per month from different bulls. The results obtained show that in the individual cases one property of semen can be correlated to fertility during one period, and another property during a second period. We must look upon the studied characteristics of semen as being derived from a number of processes in the sexual apparatus which are significant in maintaining maximal fertility. In order to obtain a true picture of the fertilizing ability of a bull thus only several different tests can give a reliable result.

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SUMMARY

The production of male sperm antagglutin in bulls and rabbits has been analysed, and the obtained figures connected to the season and to the fertility of the individuals studied. Besides the total production of the accessory glands, estimated as ejaculated volumes and amounts of solids in the semen, has been registered.

Rabbits:

1. In rabbits all properties of semen studied are at maximum during the winter and at a minimum during May and September with a secondary maximum in the middle of the summer.

Bulls:

2. In bulls the highest values of the properties studied as well as of the fertility are found during August and September, and the lowest during February.

3. One of the seven bulls shows throughout the year a good correlation between fertility and production of antagglutin. Four other bulls show such correlations during part of the year, whereas the two remaining bulls, which have a significantly lower fertility than the others, show no correlation between

fertility and production of antagglutin. Moreover, in some cases there exist correlations between fertility and some of the other properties studied.

4. Normal amounts of antagglutin are found in ejaculates rejected for artificial insemination on account of agglutination of the sperm cells, but some observations indicate that in these ejaculates the antagglutin is more or less inactivated.

5. The amounts of antagglutin are significantly correlated to those of total solids in the same ejaculates, but not to the amounts of fructose.

A high mass-activity in the ejaculates is connected with a high production of antagglutin, whereas no such relation exists with the motility of the spermatozoa. Besides in two bulls a high density of spermatozoa is accompanied by a high production of antagglutin.

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